TECHNICAL QUALIFICATION BOARD (TQB) PACKAGE

Candidate: Dr. Pamela D. Noyes

Position: Biologist/Toxicologist, US EPA, ORD, NCEA, IRIS Division

Hiring Manager: Dr. Vincent Cogliano

Package Contents:

A. **Curriculum vitae (CV)**

B. **Letters of Reference (provided separately)**

C. **Peer Review Publications**

D. **Major Scientific/Technical contributions**

Personal Note:

Thank you for considering my TQB package in support of a position with the NCEA IRIS Program. There are a lot of highly skilled and committed scientists working in the group, and NCEA more broadly, and I would consider myself lucky to have the opportunity to help them develop and achieve their goals. I have been blessed to work with and learn from some truly great people, and consider my success and progress a representation and direct result of these many collaborations, and of course a lot of hard work. The environmental challenges we face are immense but not impossible to address. I hope to continue playing a part in efforts to make a difference in the health of people and our environment. This core principle has always guided my professional and personal choices, and my sense is that there will be many opportunities to make this difference with NCEA.

SECTION A

Curriculum vitae (CV)

Pamela D. Noyes

PAMELA D. NOYES

U.S. EPA, Office of Chemical Safety and Pollution Prevention (OCSPP),
Office of Science Coordination and Policy (OSCP), Washington, DC 20460
Ph: 202.564.3043 ~ Email: noyes.pamela@epa.gov

EDUCATION

Ph.D., Duke University, Toxicology and Chemistry, Certificate in Toxicology, 2013

M.S., Johns Hopkins University, Environmental Science, Honors, 2002

Coursework, chemistry, biological sciences, ecology, modeling, statistics

George Mason University, 2003-05
Montgomery College, 1992-94

B.S., University of Maryland, Finance, 1990

HONORS AND AWARDS

2016 Best Postdoctoral Publication Award, Society of Toxicology
Ruth L. Kirschstein NIH/National Research Service Award, Postdoctoral Trainee, Oregon State, 2013-15
EPA STAR Graduate Fellowship, U.S. EPA, 2010-13
Åke Bergman and Bo Jansson Award, Excellence in Presentations, BFR Meeting, 2013
Hutzinger Award, Outstanding Student Presentation, Dioxin Meeting, 2010
Promotion, GS-14, U.S. EPA/ORD/OSP, 2002

PROFESSIONAL EXPERIENCE

Toxicologist/Biologist (GS-14; 40 hrs/wk) **01/2016 – Present**
U.S. Environmental Protection Agency
Office of Chemical Safety and Pollution Prevention (OCSPP)
Office of Science Coordination and Policy (OSCP)
Endocrine Disruptor Screening Program (EDSP)
Washington, DC

Work with EPA ORD laboratories, program offices, and NIEHS to review in vitro and in vivo studies and develop methods to evaluate chemicals for effects on the endocrine systems of humans and wildlife

Leading the development of EPA's framework for screening and testing chemicals for thyroid disruption, including approaches for integrating in vitro high-throughput screening (HTS) assays into chemical hazard and risk assessments

Managing the design and conduct of a large scientific review of in vitro and in vivo studies to identify reference chemicals and potency values to characterize chemical effects on the thyroid system

EDSP representative on OECD Test Guidelines Program workgroups, including the Validation Management Group for Ecotoxicity Testing (VMG-eco) and VMG for non-animal testing (NA)

EDSP representative on the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) under the NIEHS National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to develop and implement new and revised toxicity tests for risk assessment

Assist in managing the technical direction of contracts under the EDSP screening battery that require a suite of in vitro and in vivo mammalian, fish, and amphibian toxicity assays

Worked with colleagues in OSCP to design approaches using adverse outcome pathway (AOP) frameworks to integrate in vitro HTS assays with in vivo data generated under the EDSP screening battery to facilitate evaluating chemicals for estrogen and androgen pathway bioactivity

Represent EPA's Endocrine Disruptor Screening Program (EDSP) science and policy positions in meetings with external stakeholders and EPA program office and ORD partners

Environmental Health Scientist (40 hrs/wk) **05/2015 – 12/2015**
Chevron Energy Technology Company
Houston, TX

Identified research opportunities, managed contracts, and provided technical guidance to support Chevron's environmental planning, including proposals to use zebrafish HTS platforms for toxicity testing

Prepared risk assessments and fate and effects studies for site remediation under RCRA, CERCLA, and state regulations; assisted in planning, fieldwork oversight, and data review

Conducted a technical review of lead and mercury dose-response models for use in risk assessment

Evaluated the human health and environmental safety of several fire retardant foams to inform the selection criteria for broad-scale use on Chevron oil and gas platforms

Participated on multi-disciplinary teams in regions where gaps exist in environmental regulations, including efforts to assess/mitigate risks to metals leachate from pipelines in developing Asian countries

Coordinator, Ecological Risk Assessment Forum(GS-14; 40 hrs/wk) **12/2003 – 05/2006**
U.S. EPA, Office of Research & Development (ORD)
National Center for Environmental Assessment (NCEA)
Washington, DC

Coordinated and participated on multi-disciplinary teams to develop EPA guidance on high impact ecological and human health risk issues for chemicals. Major projects included:

- Human health and environmental risk assessment framework for metals
- Eco-risk methods for applying toxic equivalency factors (TEFs) for PCBs, dioxins, and furans
- White papers on: population level eco-risk assessment, criteria for evaluating chemicals for persistence, bioaccumulation, and toxicity (PBT), and extrapolating data across different levels of biological organization

Developed dose-response assessments for human and wildlife health to support the metals risk assessment framework

Identified and synthesized data and information from academia, industry, and the government for use in guidance to understand chemical exposure and effects relationships

Managed contracts to support development of the metals risk framework; certified as ORD project officer for Assistance Agreements, external contracting, and peer review

Officiated and organized external scientific workshops to deliberate on forum projects

Worked with the EPA Science Advisory Board (SAB) to carry out formal review of the scientific underpinnings of the metals risk assessment framework

Engaged with external government partners, including OMB, DOE, and DOD, to ensure their input in decision-making and guidance development

Regularly briefed senior EPA management on project status, schedule, and issues

Environmental Scientist (GS-14 (06/2002)/GS-13; 40 hrs/wk) **09/2001 – 12/2003**
U.S. EPA, ORD
Office of Science Policy (OSP)
Washington, DC

Managed ORD's involvement in policy and regulatory development by the EPA Office of Water (OW) and Office of Pesticide Programs (OPP)

Represented ORD and participated on workgroups with program offices and ORD science and policy staff to develop EPA policies, regulations, guidance on water- and pesticide-related issues

Spearheaded ORD's participation in OW's regulation of concentrated animal feeding operations (CAFOs) under the CWA

Coordinated ORD's input on OPP's cumulative and aggregate risk policies for pesticides under FIFRA and the FQPA

Harmonized and prepared written summaries of ORD technical and policy positions on clean water and pesticide regulations and guidances

Presented ORD's positions on clean water and pesticide regulations and guidances in workgroup meetings and to senior management

Worked with ORD and OW scientists and policy staff to:

- develop white papers on CWA and SDWA issues linked to CAFOs, notably analyses of water and food borne illness data
- design risk-hazard screening methods, including GIS mapping to assess point source discharges, for use in establishing CWA Effluent Limitation Guidelines (ELGs)
- expand phylogenetic mapping techniques to model the potential spread of invasive fish species for use in establishing ELGs for aquaculture operations
- Assist OW in preparing environmental benefits assessments under the ELG program for cooling water intake structures, construction/development sector, and meat handlers
- Examine research gaps and data needs under EPA's TMDL program to support watershed assessments

Chemical Review Manager (GS-13; 40 hrs/wk) **09/1998 – 09/2001**
U.S. EPA, Office of Pesticide Programs (OPP)
Special Review and Reregistration Division (SRRD)
Arlington, VA

Managed chemical safety evaluations of pesticides, including atrazine, terbufos, and carbofuran, subject to Special Review and reregistration under FIFRA, FFDCA, and FQPA. Involved working on multi-disciplinary teams of regulators, scientists, and economists; Responsibilities included:

- Preparing human health and ecological risk assessments, including dose-response assessments, of pesticide effects on human and wildlife health
- Preparing reregistration eligibility documents (REDs) summarizing human and wildlife risk- and benefit-based management decisions for pesticides
- Reviewing and synthesizing biological effects data from industry, government, and academia

- Developing strategies and worked with chemical companies to reduce risk from pesticides
- Working with Office of Water and registrants to ensure satisfaction with SDWA Maximum Contaminant Levels (MCLs) for pesticides
- Regularly meeting with industry, trade associations, and environmental groups to discuss and address pesticide use and risk issues
- Responding to comments/inquiries from industry, trade associations, environmental groups, the public, and press on pesticide science and policy issues
- Presenting EPA positions and regulatory decisions at public meetings and briefings
- Preparing correspondence, briefing summaries, and federal register notices; Regularly briefed senior management on project status and issues of concern

Biologist (40 hrs/wk) **10/1993 – 09/1998**
Jellinek, Schwartz & Connolly, Inc.
Arlington, VA

Worked with scientists and regulatory specialists to develop and implement integrated environmental health and safety standards for S.C. Johnson to comply with TSCA and FIFRA

Assisted with the preparation and submission of pesticide registration applications (under FIFRA, FFDCA, FQPA) and PMNs (under TSCA)

Reviewed environmental health effects studies placed with contract laboratories to support pesticide registrations; assisted in developing dose-response assessments

RESEARCH EXPERIENCE

Postdoctoral Research Fellow **10/2013 – 06/2015**
Oregon State University, Corvallis, OR
Environmental & Molecular Toxicology
Laboratory of Dr. Robert Tanguay

Research Activities –

Assisted in the design and implementation of the labs automated zebrafish HTS platforms and custom built tools for developmental toxicity and behavioral testing

Completed a large study to characterize flame retardant effects on vertebrate development using zebrafish HTS platforms to measure initiating events leading to morphological deformities and maladaptive behavior

Collaborated with internal and external colleagues to develop methodologies and custom scripts in R language to evaluate, distill, and present multi-outcome zebrafish HTS data

Assisted in the development of a predictive endocrine bioactivity framework using the zebrafish HTS platform and whole genome transcriptomic/microarray profiling

Participated in a study to characterize the modes of action of the antimicrobial agent triclosan by anchoring adverse phenotypes in zebrafish to transcriptomic changes using whole genome microarray

Initiated examination of chemical-induced mechanisms of disrupted thyroid bioactivity and developmental toxicity in zebrafish using HTS platforms, behavior assays, PCR, and gene-specific oligonucleotide (morpholino) knockdown approaches

Mentored and trained students in professional career-academic interests, laboratory research methods, and standard operating procedures, and served as a thesis committee member

Doctoral Research Fellow **08/2006 – 05/2013**
Duke University, Durham, NC
Integrated Toxicology and Environmental Health Program
Laboratory of Dr. Heather Stapleton

Research Activities –

Designed and conducted toxicity studies to characterize the uptake, metabolism, and endocrine disrupting effects of flame retardant chemicals on different life-stages of fishes

Developed and published new methods to measure circulating thyroid hormones in fish using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS)

Routinely conducted RNA extractions and qPCR; applied degenerate primer design to sequence partial cDNAs of deiodinases in the fathead minnow; sequences published in NCBI GenBank

Developed and published methods using LC/MS/MS to measure the activity of deiodinase enzymes in different organ systems of fish, including regular isolation of microsomal and S9 fractions

Experienced in histological examinations of the fish thyroid and liver; identified new histopathology in livers of developing fish exposed to PBDEs

Measured and published effects of PBDEs on reproductive endpoints (gonadal somatic index; GSI) of adult male fathead minnows using published EPA methods

Prepared and published an in-depth review of flame retardant effects on the thyroid and reproductive systems of biota, and the potential cross-talk between these pathways

Prepared sediment, water, and tissue samples for analysis of a variety of environmental contaminants, including PBDEs, PCBs, pesticides, and other organic substances

Conducted chemical analysis of sediment, biosolid, and tissue samples using gas chromatography mass spectrometry (GC/MS) and LC/MS/MS; analyzed data using ChemStation and MassHunter software

Constructed a large fish culture system to house and breed fathead minnows for toxicity testing; assisted in the design and setup of the labs zebrafish colony system

Assisted in sampling houses and children for flame retardants as part of Dr. Stapleton's NIH R01 grant to examine flame retardant exposures and thyroid effects among children

Employed radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) methods to measure circulating thyroid hormones in humans, rodents, and fish

Trained and mentored undergraduate and fellow graduate students in laboratory chemistry and toxicology research, fish culturing methodologies, and standard operating procedures

Provided advice to other laboratories and scientists on methods developed for measuring the effects of thyroid hormone disrupting PBDEs on fishes

Made presentations to visiting faculty, contributors, and senior department/graduate school officials on lab research programs and results

ASSISTANCE TO SCIENTIFIC COMMUNITY

Invited Guest Editor, Special Issue, Journal of Current Zoology, 07/2014 – 09/2015

Collaborative series of papers devoted to the impacts of chemical exposures and climate change on wildlife health with special focus on endocrine disrupting chemicals

Invited Book Chapter Co-author, Comprehensive Toxicology, 3rd Edition, 05/2015 – Present

Noyes PD, Garcia GR, Tanguay RL. 2016. Zebrafish in developmental toxicology: Linking genetics, "omic" technologies, behavior and high throughput testing [In review].

SETAC Pellston Workgroup, 02/2011-02/2013

Developed approaches using Adverse Outcome Pathway (AOP) frameworks to evaluate the influence of climate change on chemical toxicity; developed an AOP to describe interactive effects of thyroid disrupting chemicals and climate change on amphibian populations

Invited Co-author, United Nations Environment Programme (UNEP), Norwegian EPA, 06/2013–12/2013

Prepared review and analysis of bioaccumulation potential of the PBDE flame retardant DecaBDE for listing under the Stockholm Convention on Persistent Organic Pollutants (POPs)

Journal Peer Review

Regularly peer review toxicology and chemistry studies submitted to scientific journals; prepare written reviews, including recommendations for acceptance, revisions, and additional testing

Volunteer Diver, National Aquarium in Baltimore, 1999-2005

Prepared food and conducted in-water feedings of animals in coral reef and ray fish exhibits; Presented to student groups and public on coral reef and estuarine exhibits/ecosystems; one full day every other week

PEER-REVIEW PUBLICATIONS

Noyes PD, Garcia GR, Tanguay RL. 2016. Zebrafish as an in vivo model for sustainable chemical design. *Green Chem* 18:6410-6430.

Browne P, Noyes PD, Casey WM, Dix DJ. 2016. Evaluating endocrine activity of chemicals using adverse outcome pathways. *Environ Health Perspect* [In Review].

Haggard DE, Noyes PD, Waters KE, Tanguay RL. 2016. Phenotypically anchored transcriptome profiling of developmental exposure to the antimicrobial agent, triclosan, reveals hepatotoxicity in embryonic zebrafish. *Toxicol Appl Pharm* 308:32-45.

Garcia GR, Noyes PD, Tanguay RL. 2016. Advancements in zebrafish applications in 21st century toxicology. *Pharmacol Ther* 161:11-21.

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. 2015. Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to halogenated and organophosphate flame retardants. *Toxicol Sci* 145(1): 177-195.

Noyes PD and Lema SC. 2015. Editorial: Heating up the environmental context of chemical pollution: Ecotoxicology in a changing global climate. *Curr Zool* 61(4): 614-616.

Noyes PD and Lema SC. 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. *Curr Zool* 61(4): 669-689.

Noyes PD, Stapleton HM. 2014. PBDE flame retardants: Toxicokinetics and thyroid endocrine disruption in fish. *Endo Disruptors* 2: 2943001-2943025.

Noyes PD, Lema SC, Roberts SC, Cooper EM, Stapleton HM. 2014. Rapid method for the measurement of circulating thyroid hormones in low volumes of fish plasma by LC-ESI/MS/MS. *Anal Bioanal Chem* 406(3): 715-726.

Muzzio AM, Noyes PD, Stapleton HM, Lema SC. 2014. Tissue distribution and thyroid hormone effects on mRNA abundance for membrane transporters Mct8, Mct10, and organic anion-transporting polypeptides (Oatps) in a teleost fish. *Comp Biochem Physiol A* 167:77-89.

Noyes PD, Lema SC, Macaulay LJ, Douglas NK, Stapleton HM. 2013. Low level exposure to the flame retardant BDE-209 reduces thyroid hormone levels and disrupts thyroid signaling in fathead minnows. *Environ Sci Technol* 47(17):10012-10021.

Hooper MJ, Ankley GT, Cristol DA, Maryoung LA, Noyes PD, Pinkerton KE. 2013. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. *Environ Toxicol Chem* 32(1):32-48.

Noyes PD, Hinton DE, Stapleton HM. 2011. Accumulation and debromination of decabromodiphenyl ether (BDE-209) in juvenile fathead minnows (*Pimephales promelas*) induces thyroid disruption and liver alterations. 2011. *Toxicol Sci* 122(2):265-274.

Roberts SC, Noyes PD, Gallagher EP, Stapleton HM. 2011. Species-specific differences and structure-activity relationships in the debromination of PBDE congeners in three fish species. *Environ Sci Technol* 45(5):1999-2005.

Noyes PD, McElwee MK, Miller HD, Clark BW, Van Tiem LA, Walcott KC, Erwin KN, Levin ED. 2009. The toxicology of climate change: Environmental contaminants in a warming world. *Environ Int* 35(6):971-986.

Noyes PD, Kelly SM, Mitchelmore CL, Stapleton HM. 2010. Characterizing the *in vitro* hepatic biotransformation of the flame retardant BDE-99 by common carp. *Aquatic Tox* 97(2):142-150.

Timme-Laragy AR, Noyes PD, Buhler DR, Di Giulio RT. 2008. CYP1B1 knockdown does not alter synergistic developmental toxicity of polycyclic aromatic hydrocarbons in zebrafish. *Mar Environ Res* 66(1):85-87.

SCIENTIFIC PRESENTATIONS

Seminars

09/2015. US EPA, Office of Chemical Safety and Pollution Prevention, Office of Science Coordination and Policy. Topic: Flame retardants: Predicting biological effects using fish models.

01/2015. Oregon State University, Superfund Research Program Colloquium. Topic: Use of advanced testing methods in zebrafish to examine the developmental toxicity of flame retardants and structure-activity relationships

12/2014. Chevron Energy Technology Company. Topic: Using zebrafish as a biological sensor to characterize chemical bioactivity and improve environmental performance

05/2013. Oregon State University, Corvallis, OR. Topic: PBDE Flame Retardants: Accumulation, metabolism, and disrupted thyroid regulation in fish

04/2013. Duke University, Durham, NC. Topic: PBDE Flame Retardants: metabolism and disrupted

thyroid regulation in early and adult life stages of fish.

02/2012. US EPA, Mid-Continent Ecology Division, Duluth, MN. Topic: Interactive effects of climate change and contaminant exposures on toxicity and wildlife health

Meeting Presentations

SOT, New Orleans, LA, 3/2016. Phenotypically-anchored transcriptomic response in embryonic zebrafish developmentally exposed to triclosan [Poster]

SOT, San Diego, CA, 3/2015. Flame retardants: Advanced morphological-behavioral test platform using zebrafish reveals developmental defects [Poster]

Flame Retardants Meeting, San Francisco, CA, 4/2013. Low-level exposures to BDE-209 reduce thyroid hormones and disrupt thyroid signaling in fish [Platform]

SETAC, Boston, MA, 11/2011. SETAC Pellston results: Mechanistic toxicology in the face of climate change [Platform]

SETAC, Portland, OR, 11/2010. Effects of BDE-209 on thyroid regulation in early life stages of fish [Platform]

Dioxin Symposium, San Antonio, TX, 9/2010. PBDE effects on thyroid regulation in early life stages of fish [Platform]

SETAC, New Orleans, LA, 11/2009. Characterizing PBDE metabolism in fish [Poster]

Flame Retardants Meeting, Ottawa, ON, 5/2009. Debromination of BDE-209 by fathead minnow [Poster]

TEACHING

Instructor, Scientific Skills and Ethics, Oregon State University, 2014
Instructor, Introductory Chemistry & Toxicology; Principles of Endocrine Disruption, Duke, 2010-11
Teaching Assistant, Chemical Fate of Organic Compounds, Duke, 2006-07
Teaching Assistant, Environmental Toxicology & Chemistry, Duke, 2006-07
Teaching Assistant, Estuarine and Coastal Ecology, George Mason, 2005

THESIS COMMITTEES (Honor Undergrads)

Kimberly Britsch, Oregon State University, Biology Honors, 2014
Cory Gerlach, Oregon State University, Toxicology & Biotechnology, Honors College, 2014

PROFESSIONAL ASSOCIATIONS

Society of Environmental Toxicology and Chemistry (SETAC)
Society of Toxicology (SOT)

SECTION B

Letters of Reference for Pamela D. Noyes

Provided directly to Dr. Vincent Cogliano under separate cover

SECTION C

Major Peer Review Publications

Pamela D. Noyes

Peer Review Publication 1:

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. 2015. Advanced morphological-behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to halogenated and organophosphate flame retardants. *Toxicological Sciences* 145(1): 177-195.

Basis for inclusion and scientific impact:

Millions of pounds of flame-retardants (FRs) are used every year in consumer products and industrial applications with the intent of preventing fire-related casualties. FR chemicals include a variety of chemical structures, and biomonitoring data in humans and wildlife show bioaccumulation of a number of these chemicals. Like many of the tens of thousands of chemicals used today, they have been deployed into products with limited understanding of their toxicity potential.

Dr. Robert Tanguay's laboratory at Oregon State University, where I was working under a NIH/NRSA postdoctoral fellowship, has been a global leader in developing embryonic zebrafish high-throughput screening (HTS) assay technologies. This study conducted in Dr. Tanguay's lab is the first to use zebrafish HTS assays to test a large suite of diverse FR chemical structures in tandem by measuring numerous morphological and behavioral outcomes. Over 90% of the chemicals tested were bioactive at one or more concentrations and endpoints evaluated. These data have not only been critical in characterizing our understanding of FR bioactivity, but have played a major role in validating the reliability of zebrafish HTS platforms and computational toxicology approaches to evaluate multi-dimensional data outputs. It is the first study to incorporate novel photomotor response (PMR) behavioral testing of FRs in the very earliest life-stages of zebrafish development when neurological structural patterning is being initiated. Another major contribution of the study was development of computational approaches to understand and compare chemical structure and bioactivity relationships. We were able to group and identify structural features of the FRs tested that impaired normal development with high potency. Thus, this platform approach is significant because it allows for a quantitative tool to design inherently safer chemicals that confer less bioactivity.

I was the lead in study design, conduct, trouble-shooting, and R coding and data analysis. I worked closely with internal coauthors, including Dr. Tanguay, my postdoctoral mentor, and consulted with external colleagues, to design and carryout the multi-dimensional data analyses. I determined major findings from the study, prepared the manuscript, and managed the peer review.

I was awarded SOT's Best Postdoctoral Publication Award in 2016 for this work. I presented the study results at the SOT meeting in 2016, including at a luncheon awards ceremony. This was one of the featured articles by the Editor of Toxicological Sciences at its publication (http://toxsci.oxfordjournals.org/content/145/1/1.full). It was also highlighted by EPA in March 2016 on the agency's external blog site (https://blog.epa.gov/blog/2016/03/one-fish-two-fish-test-fish-control-fish-award-winning-high-throughput-research-on-flame-retardants-in-zebrafish/).

TOXICOLOGICAL SCIENCES, 145(1), 2015, 177–195

doi: 10.1093/toxsci/kfv044
Advance Access Publication Date: February 23, 2015

Advanced Morphological — Behavioral Test Platform Reveals Neurodevelopmental Defects in Embryonic Zebrafish Exposed to Comprehensive Suite of Halogenated and Organophosphate Flame Retardants

Pamela D. Noyes, Derik E. Haggard, Greg D. Gonnerman, and Robert L. Tanguay[1]

Department of Environmental & Molecular Toxicology, Environmental Health Sciences Center, and the Sinnhuber Aquatic Research Laboratory, Oregon State University, Corvallis, Oregon 97331

[1]To whom correspondence should be addressed. Fax: 541-737-6074. E-mail: robert.tanguay@oregonstate.edu.

ABSTRACT

The increased use of flammable plastics and electronic devices along with stricter fire safety standards has led to the heavy use of flame retardant chemicals in many consumer, commercial, and industrial products. Although flame retardant use has increased, a great deal of uncertainty surrounds their safety with some evidence showing toxicity and risk to human and environmental health. Recent efforts have focused on designing high-throughput biological platforms with nonmammalian models to evaluate and prioritize chemicals with limited hazard information. To complement these efforts, this study used a new morphological and behavioral testing platform with embryonic zebrafish to characterize the developmental toxicity of 44 halogenated and organophosphate flame retardants, including several of their known metabolites. Zebrafish were exposed to flame retardants from 6 to 120 h post fertilization (hpf) across concentrations spanning 4 orders of magnitude (eg, 6.4 nM to 64 μM). Flame retardant effects on survival and development were evaluated at 24 and 120 hpf, and neurobehavioral changes were measured using 2 photomotor response (PMR) assays. Compared to controls, 93% (41/44) of flame retardants studied elicited adverse effects among one or more of the bioassays and concentrations tested with the aryl phosphate ester (APE)-based mono-isopropylated triaryl phosphate and the brominated-bisphenol-A analog tetrabromobisphenol-A producing the greatest array of malformations. Hierarchical clustering showed that APE flame retardants with isopropyl, butyl, and cresyl substituents on phenyl rings clustered tightly and were particularly potent. Both PMR assays were highly predictive of morphological defects supporting their use as nonlethal means of evaluating teratogenicity that could allow for additional evaluations of long-term or delayed effects in older animals. Taken together, evidence presented here indicates that zebrafish neurodevelopment is highly sensitive to many flame retardants currently in use and can be used to understand potential vulnerabilities to human health.

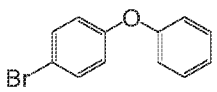
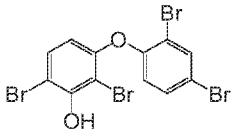
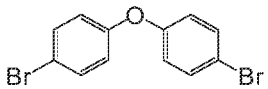
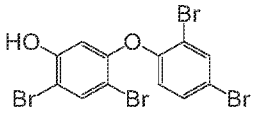
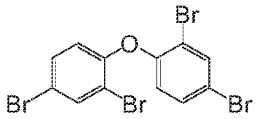
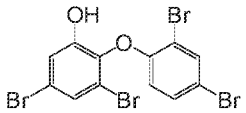
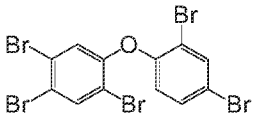
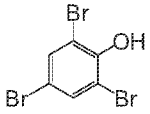
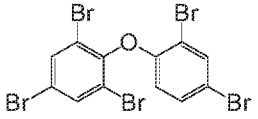
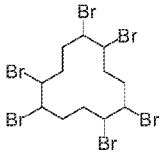
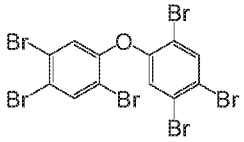
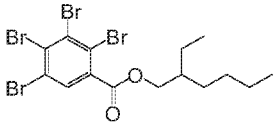
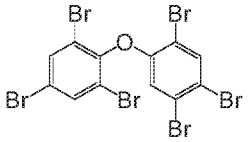
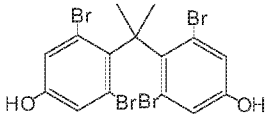
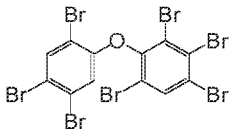
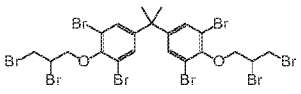
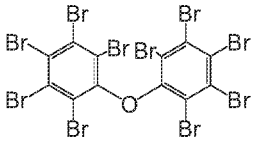
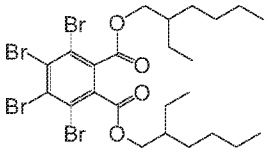
Key words: Firemaster 550; neurotoxicity; PBDE; TBBPA; TDCPP; TCPP; TCEP; TPP; teratogenicity

Dramatic increases in the use of flammable plastics and electronics coupled with stricter fire safety standards have resulted in the heavy use of flame retardant chemicals. Flame retardants today represent a diverse array of chemicals with differing structural characteristics and fire suppressing properties. Increased public, media, and government scrutiny of flame retardants in recent years has called attention to their design, use, and safety. Two of the commonly used classes of flame retardants, brominated flame retardants (BFRs) and organophosphate-based flame

retardants, which may include both halogenated and nonhalogenated structural forms, are widely used in a variety of consumer products, such as furniture, textiles, electronics, and building materials.

Polybrominated diphenyl ether (PBDE) flame retardants (Table 1) were additive BFRs used in furniture and electronic products. They were marketed as 3 major commercial mixtures: PentaBDE, OctaBDE, and DecaBDE. PentaBDE was a heterogeneous mixture of tetra-, penta-, and hexaBDEs that was added mostly to polyurethane foams and textiles, and to a lesser extent in epoxy and phenolic resins and polyesters. The vast majority (approximately 95%) of PentaBDE was used in North America (United States and Canada) in the manufacture of polyurethane foams in cushioning. As a result of this greater use in

TABLE 1. Structures of Targeted BFRs

Abbreviations	Name/CAS no	Structure	Abbreviations	Name/CAS no	Structure
BDE-3	*p*-bromodiphenyl ether [101-55-3]		3-OH-BDE-47*	3-hydroxy-2,2′4,4′-tetrabromodiphenyl ether	
BDE-15	*p,p*-dibromodiphenyl ether [2050-47-7]		5-OH-BDE-47*	5-hydroxy-2,2′4,4′-tetrabromodiphenyl ether	
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether [5436-43-1]		6-OH-BDE-47*	6-hydroxy-2,2′4,4′-tetrabromodiphenyl ether	
BDE-99	2,2′,4,4′,5-pentabromodiphenyl ether [60348-60-9]		2,4,6-TBP*	2,4,6-tribromophenol [118-79-6]	
BDE-100	2,2′,4,4′,6-pentabromodiphenyl ether [189084-64-8]		HBCD	Hexabromocyclododecane (Commercial substance) [3194-55-6]	
BDE-153	2,2′,4,4′,5,5′-heptabromodiphenyl ether [68631-49-2]		TBB	2-ethylhexyl-tetrabromobenzoate [183658-27-7]	
BDE-154	2,2′,4,4′,5,6′-hexabromodiphenyl ether [207122-15-4]		TBBPA	3,3′,5,5′-tetrabromobisphenol A [79-94-7]	
BDE-183	2,2′,3,4,4′,5,5′-heptabromodiphenyl ether [68631-49-2]		TBBPA-DBPE	Tetrabromo-bisphenol A-2,3-dibromopropyl ether [21850-44-2]	
BDE-209	Decabromodiphenyl ether [1163-19-5]		TBPH	Bis(2-ethylhexyl)-tetrabromophthalate [26040-51-7]	

Metabolites are denoted with asterisks.

North America, PentaBDE congeners have been detected at higher levels in the U.S. population than in European and Asian populations (Johnson-Restrepo and Kannan, 2009; Toms *et al.*, 2011). The production and use of Penta and OctaBDE has been phased out in the United States and banned in the EU since the mid-2000s due to concerns about their persistence, bioaccumulation, and toxicity. In 2009, these products were also listed as persistent organic pollutants under the UN Stockholm Convention (UNEP, 2009). DecaBDE contains the fully brominated congener decabromodiphenyl ether (BDE-209; approximately 97%) with trace amounts of nonaBDEs. It is an additive in high impact polystyrene, polyolefins, and polypropylene used in electronic equipment (eg, plastic housing), automobiles, airplanes, construction and building materials (eg, wires, cables, pipes), and textiles. In the United States, DecaBDE was subject to a voluntary phase-out at the end of 2013. It has also been restricted from use in electrical and electronic equipment since 2008 under the EU Restriction of Hazardous Substances Directive. At present, DecaBDE is not subject to restrictions in any Asian countries.

With the phase-out of Penta and OctaBDEs, decreasing trends or a leveling off of some PBDEs are now being measured in some biota and environmental media (Law *et al.*, 2014). Nonetheless, constituents of PentaBDE, including BDE-47 (2,2′4,4′-tetraBDE), BDE-99 (2,2′,4,4′,5-pentaBDE), BDE-100 (2,2′,4,4′,6-pentaBDE), BDE-153 (2,2′,4,4′,5,5′-hexaBDE), and BDE-154 (2,2′,4,4′,5,6′-hexaBDE), continue to be dominant PBDEs detected in humans and wildlife worldwide despite the generally more limited use of PentaBDE outside the United States (Law *et al.*, 2014). Sources of these congeners are likely related to the ongoing use and recycling of products that contain PentaBDE as well as their high environmental persistence and long-range global transport potential (de Wit *et al.*, 2010). Another source of lower MW PBDEs may be attributable to the breakdown of higher PBDEs, such as BDE-209 which can undergo photolytic (Stapleton and Dodder, 2008) and metabolic breakdown (Noyes *et al.*, 2011; Stapleton *et al.*, 2004) to yield lower MW congeners. BDE-209 is now the dominant PBDE measured in abiotic compartments, typically at ppb to low ppm levels in dust (Stapleton *et al.*, 2012a), soils and sediments (Marvin *et al.*, 2013), and biosolids (Peng *et al.*, 2009). Human body burdens of BDE-209 also appear to be on the rise in some populations, notably among E-waste workers (Bi *et al.*, 2007; He *et al.*, 2013) and in the general population, particularly young children in the United States (Lunder *et al.*, 2010; Stapleton *et al.*, 2012a).

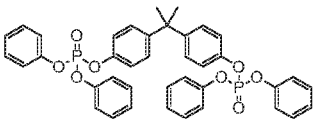
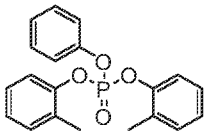
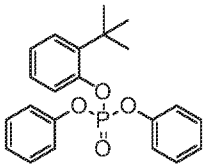
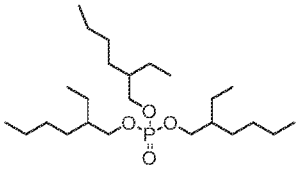
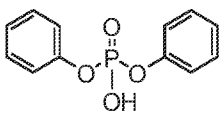
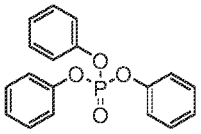
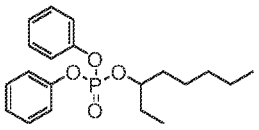
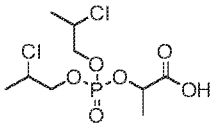
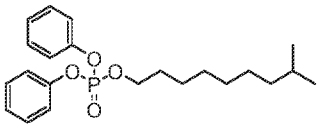
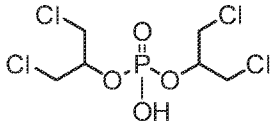
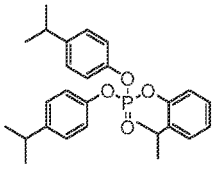
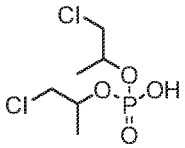
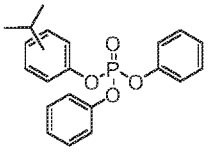
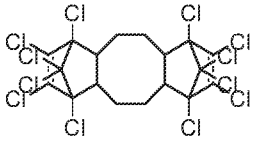
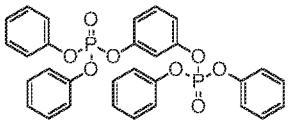
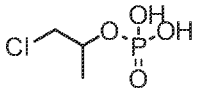
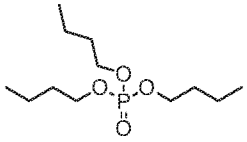
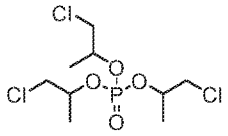
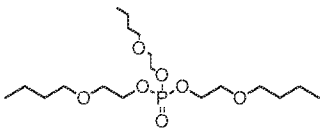
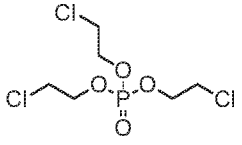
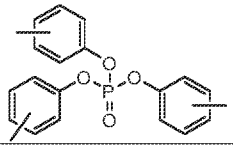
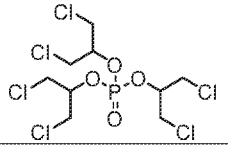
Restrictions on the use of PBDEs have resulted in the increased use of alternate flame retardants, notably organophosphate flame retardants (OPFRs; Table 2). The OPFRs have been used for many years with a diversity of applications that may extend beyond their use as flame retardants, including as plasticizers and lubricants in industrial, commercial, and consumer products. Likewise, they can also be used in a range of polymers depending on the types of side chains present. For example, nonhalogenated OPFRs, such as triphenyl phosphate (TPP) and tricresyl phosphate (TCP) may be used as flame retardant plasticizers in PVC, thermoplastics, and synthetic rubbers, with TPP also being present in the flame retardant mixture Firemaster 550 (FM550), which is a replacement for PentaBDE that is added to polyurethane foams. Similarly, the chlorinated OPFR tris (1,3-dichloro-2-propyl) phosphate (TDCPP) is also an important replacement for PentaBDE and is used in polyurethane foams in residential furniture. Despite the increased and varied uses of OPFRs, information on their exposure and environmental contamination profiles is still limited. Much of the literature focuses on rising levels of OPFRs in abiotic environments (Klosterhaus *et al.*, 2012; Shoeib *et al.*, 2014) and indoor environments, especially dust (Meeker *et al.*, 2013; Stapleton *et al.*, 2009; van den Eede *et al.*, 2011). More recent studies have also begun to document increasing exposures and bioaccumulation of OPFRs in humans (Butt *et al.*, 2014; Cooper *et al.*, 2011; Fromme *et al.*, 2014; Kim *et al.*, 2014) and wildlife (Greaves and Letcher, 2014; McGoldrick *et al.*, 2014; Sundkvist *et al.*, 2010).

One ongoing challenge with the production and use of flame retardants is that many, including PBDEs and most OPFRs are not incorporated into polymers until after polymerization and so are not chemically bound but are rather mixed into parent polymers. This additive practice presents exposure concerns as these compounds may volatilize into the air and migrate into surrounding environments, notably dust, with the breakdown of the parent polymer. Another important issue with many flame retardants currently used or considered as replacement options is that there is often little data available on their toxicity potential prior to deployment into products. Thus, there is a need to expand our understanding of the toxicity of flame retardants that are environmentally widespread and to better assess the suitability of chemicals being used/considered as replacements for those that have been banned or phased-out.

The zebrafish (*Danio rerio*) is an increasingly important biological sensor and model for screening chemicals for human health hazard and disease (Padilla *et al.*, 2012; Perkins *et al.*, 2013; Truong *et al.*, 2014). They are small prolific spawners that are easy to manipulate genetically and pharmacologically (Granato *et al.*, 1996; Howe *et al.*, 2013). Moreover, the zebrafish and mammalian brain share many anatomical and functional features, including well-conserved neuronal morphology and neurotransmitter systems, although neuroanatomical differences exist between fishes and mammals (eg, comparatively small telencephalon in fish that lack characteristic structures of a cerebral cortex) (Kalueff *et al.*, 2014; Panula *et al.*, 2010). A number of neurobehavioral tests of locomotion, anxiety, and exploration have been modeled in zebrafish, and increasing evidence appears to support well-conserved responses resembling those of rodents (Champagne *et al.*, 2010; Panula *et al.*, 2006). For instance, zebrafish display anxiety-like behaviors, such as dark avoidance and thigmotaxis, when placed in novel test environments and these responses are consistent with observations in rodents, thereby providing promising approaches for evaluating chemical hazard in nonmammalian models (Champagne *et al.*, 2010; Levin *et al.*, 2007; Steenbergen *et al.*, 2011). To this end, our laboratory and others have designed high-throughput methodologies with embryonic zebrafish to rapidly screen chemicals for neurological and developmental toxicity. These types of screening platforms have greatly expanded our capacity to assess large chemical libraries for teratogenicity, including for example those under the U.S. EPA ToxCast program (Padilla *et al.*, 2012; Truong *et al.*, 2014). They provide a systematic means of testing large, structurally diverse classes of compounds like the flame retardants. Importantly, these types of approaches represent an attractive option for *in vivo* screening early in R&D processes to help identify promising chemistries that elicit reduced bioactivity and abandon others with unwanted side effects.

The purpose of this study was to use this type of screening platform with embryonic zebrafish to increase our understanding of flame retardant bioactivity and toxicity potential. It had 2 major components: (1) evaluations of survival and 20 other teratogenicity endpoints in embryos at 24 and 120 hpf; and characterizations of locomotor behavior using 2 photomotor response (PMR) assay tests at 24 and 120 hpf (Fig. 1). Each flame retardant was tested across a range of nominal concentrations that

TABLE 2. Structures of Targeted APE, CPE, and Dechlorane Plus Flame Retardants

Abbreviations	Name/CAS no	Structure	Abbreviations	Name/CAS no	Structure
BDP	Bisphenol A bis-(diphenyl phosphate) [5945-33-5]		o-TCP	Tri-o-cresyl phosphate [78-30-8]	
BPDP	t-Butylphenyl diphenyl phosphate [56803-37-3]		TEHP	Tris (2-ethylhexyl) phosphate [78-42-2]	
DPP*	Diphenyl phosphate [838-85-7]		TPP	Triphenyl phosphate [115-86-6]	
EHDP	2-Ethylhexyl diphenyl phosphate [1241-94-7]		BCPCP*	Bis (2-chloropropyl) 1-carboxyethyl phosphate	
IDDP	Isodecyl diphenyl phosphate [29761-21-5]		BDCPP*	Bis(1,3-dichloro-2-propyl)phosphate [72236-72-7]	
IPP-1 IPP-2 IPP-3	Isopropylated triphenyl phosphate [68937-41-7]		BCPP*	Bis (2-chloropropyl) phosphate [789440-10-4]	
mITP	Mono isopropylated triaryl phosphate (*p*-, *m*-, o- mixture)		Dechlorane	Dechlorane Plus [13560-89-9]	
RDP	Resorcinol A bis-(diphenyl phosphate) [57583-54-7]		MCPP*	Mono (2-chloropropyl) phosphate [109827-89-6]	
TBP	Tributyl phosphate [126-73-8]		TCPP	Tris (1-chloropropyl) phosphate[13674-84-5]	
TBEP	Tris (2-butoxyethyl) phosphate [78-51-3]		TCEP	Tris (2-chloroethyl) phosphate [115-96-8]	
TCP	Tricresyl phosphate (*p*-, *m*-, o- mix) [1330-78-5]		TDCPP	Tris (1,3-dichloro-2-propyl) phosphate [13674-87-8]	

Metabolites are denoted with asterisks.

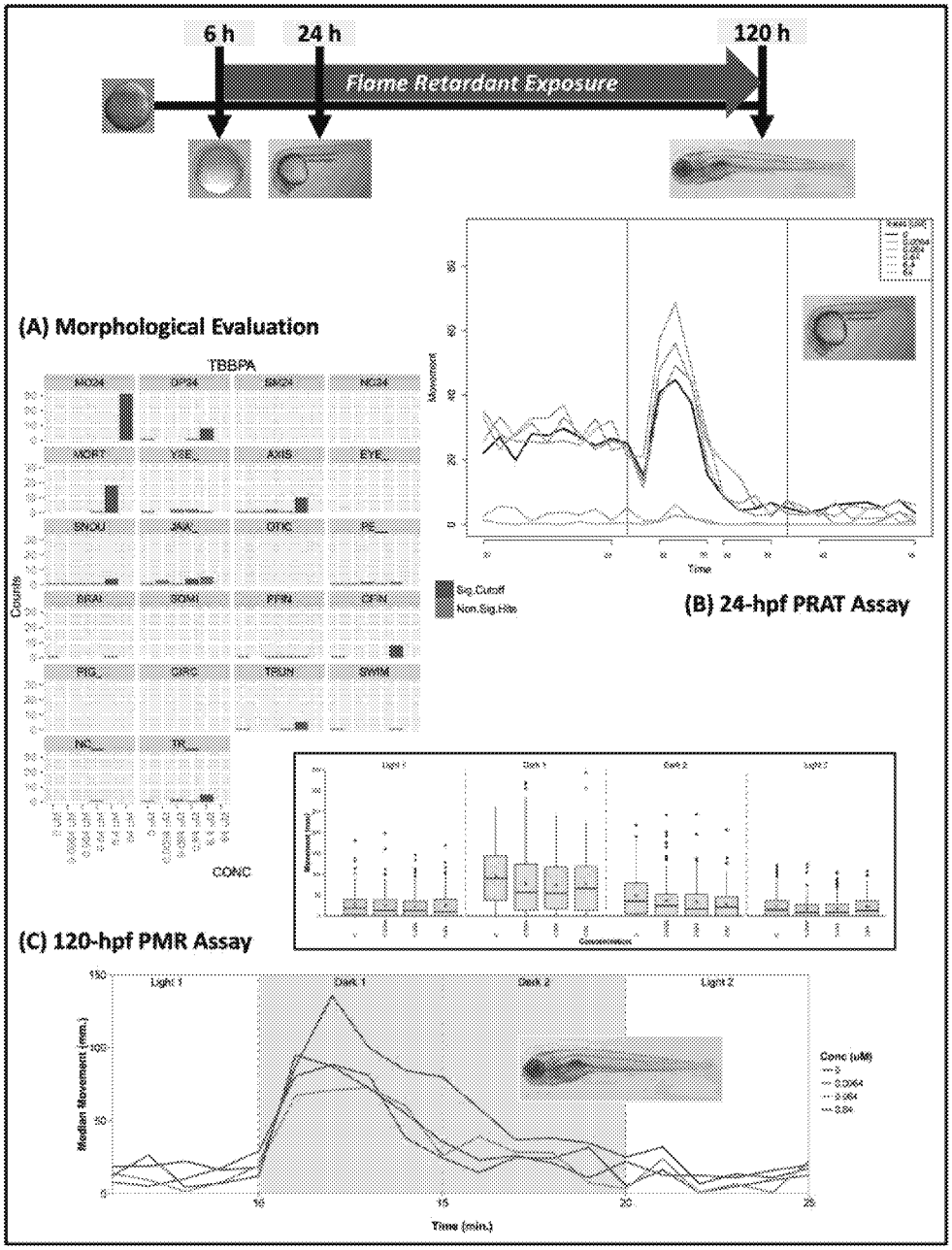


FIG 1. Schematic of A, morphological and B and C, behavioral testing platform used to characterize the effects of flame retardants on zebrafish neurological and morphological development at 24 and 120 hpf. Embryos were exposed continuously to chemical from 6 to 120 hpf under static nonrenewal conditions.

spanned 4 orders of magnitude (eg, 6.4 nM to 64 μM) and included 32 replicates at each concentration. The flame retardants tested (Tables 1 and 2) included aryl phosphate ester (APE) and chlorinated phosphate ester (CPE) OPFR chemicals that are increasing in use with still insufficient toxicity testing. A number of BFRs were also examined, including the PBDEs and heavily used flame retardants tetrabromobisphenol-A (TBBPA) and hexabromocyclododecane (HBCD; Table 1). TBBPA is the most widely used BFR with early reports of increasing global market demand ranging from 120 000 to 170 000 metric tons between 1999 and 2004 (Guerra *et al.*, 2011). It is a brominated analog of BPA that is used primarily as a reactive flame retardant in printed circuit boards and also as an additive flame retardant in polymers. HBCD is an additive brominated cyclic alkane added to polystyrene thermoplastic polymers in furniture, appliances, and construction materials. Although TBBPA has been used in greater volumes than other BFRs, reported concentrations of TBBPA in biota and the environment appear to be less than the PBDEs (ECB, 2006; Kemmlein *et al.*, 2009). However, the EU concluded in its risk characterization of TBBPA that additional information and testing were needed with some questions raised due to its primary biodegradation to several products including BPA. Components of the commercial mixture FM550, which have been frequently detected in furniture foam since the 2005 phase-out of PentaBDE, were also studied (Dodson *et al.*, 2012; Stapleton *et al.*, 2012b). Although FM550 is proprietary, studies have identified its major components as TPP, 2-ethylhexyl-tetrabromobenzoate (TBB), bis(2-ethylhexyl)-tetrabromophthalate (TBPH), and isopropylated triaryl phosphates (ITPs) (Stapleton *et al.*, 2008). Recent studies also report frequent detections

of TDCPP in indoor environments and its primary metabolite bis(1,3-dichloroisopropyl)phosphate (BDCPP) in human urine since replacement of PentaBDE, and so both these chemicals were examined (Cooper *et al.*, 2011; Meeker *et al.*, 2013). In addition to testing TDCPP and BDCPP, several other chlorinated-tris flame retardants and known metabolites were targeted.

MATERIALS AND METHODS

Chemicals. Table 3 provides a list of flame retardants tested along with suppliers, stock purity, and concentration ranges evaluated. Three different formulations of isopropylated triphenyl phosphate (IPP) were tested from different manufacturers as this chemical is a complex mixture of numerous positional isomers and the phenol groups may be mono-, di-, or tri-isopropylated. The toxicity of the polychlorinated flame retardant Dechlorane Plus was also characterized, and although not an organophosphate, it was grouped with the CPEs because of shared chlorination. A number of metabolites were also tested, including BDCPP (TDCPP metabolite), bis(2-chloropropyl)1-carboxyethyl phosphate (BCPCP; tris (2-chloroethyl) phosphate [TCEP] metabolite), diphenyl phosphate (DPP; TPP metabolite), bis(2-chloroisopropyl)phosphate and mono(2-chloroisopropyl)phosphate (BCPP, MCPP; tris

TABLE 3. Sources, Purities, and Test Concentrations ($N = 32$) of Targeted Flame Retardant Parents and Metabolites.

Flame retardant	Supplier	Purity (%)	Concentration ranges tested (μM)
APE FRs			
BDP	Toronto Research	98	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
BPDP	Ubichem PLC	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
DPP*	Sigma-Aldrich	99	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
EHDP	TCI Americas	92.8	64, 6.4, 0.64, 0.064, 0.0064, 0
IDDP	Ferro Corp	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
IPP-1	Ameribrom	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
IPP-2	Chemtura	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
IPP-3	Amfinecom Inc	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
mITP	Chemtura and Wellington	> 90	6.4, 0.64, 0.064, 0.0064,0.00064
RDP	Toronto Research	98	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TBP	Sigma-Aldrich	99	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TBEP	Chiron AS	95.5	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TCP	Acros Organics	99	64, 6.4, 0.64, 0.064, 0.0064, 0
o-TCP	Acros Organics	96.8	64, 6.4, 0.64, 0.064, 0.0064, 0
TEHP	Sigma-Aldrich	97	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TPP	Acros Organics	99.3	64, 6.4, 0.64, 0.064, 0.0064, 0
CPE FRs and Dechlorane			
BCPCP*	MRI Global	96	64, 6.4, 0.64, 0.064, 0.0064, 0
BDCPP*	Toronto Research	95	64, 6.4, 0.64, 0.064, 0.0064, 0
BCPP*	MRI Global	99.5	64, 6.4, 0.64, 0.064, 0.0064, 0
Dechlorane	Toronto Research	98	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
MCPP*	MRI Global	95.1	64, 6.4, 0.64, 0.064, 0.0064, 0
TCPP	Albemarle Corp	NP	64, 6.4, 0.64, 0.064, 0.0064, 0
TCEP	Sigma-Aldrich	98.8	64, 6.4, 0.64, 0.064, 0.0064, 0
TDCPP	Sigma-Aldrich	99	64, 6.4, 0.64, 0.064, 0.0064, 0
PBDE FRs			
DE-71 (PentaBDE commercial mix)	Great Lakes Chem	98	64, 6.4, 0.64, 0.064, 0.0064, 0
DE-79 (OctaBDE commercial mix)	Great Lakes Chem	99	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-3	Sigma-Aldrich	98.6	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-15	Sigma-Aldrich	99.6	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-47	Cerilliant Corp	96	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-99	Cerilliant Corp	96	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-100	AccuStandard	100	0.0064, 0.00064, 6.4E-05, 6.4E-06, 0
BDE-153	Cerilliant Corp	98	64, 6.4, 0.64, 0.064, 0.0064, 0
BDE-154	AccuStandard	100	0.0064, 0.00064, 6.4E-05, 6.4E-06, 0
BDE-183	AccuStandard	100	0.0064, 0.00064, 6.4E-05, 6.4E-06, 0
BDE-209	Sigma-Aldrich	99.9	64, 6.4, 0.64, 0.064, 0.0064, 0
3-OH-BDE-47*	AccuStandard	97.1	0.64, 0.064, 0.0064, 0.00064, 6.4E-05, 0
5-OH-BDE-47*	AccuStandard	98	0.64, 0.064, 0.0064, 0.00064, 6.4E-05, 0
6-OH-BDE-47*	AccuStandard	98	0.0064, 0.00064, 6.4E-05, 6.4E-06, 0
2,4,6-TBP*	Sigma-Aldrich	99.8	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
Other brominated FRs			
HBCD (commercial substance)	Sigma-Aldrich	95	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TBB	Toronto Research	96	64, 6.4, 0.64, 0.064, 0.0064, 0
TBBPA	Sigma-Aldrich	99.2	64, 6.4, 0.64, 0.064, 0.0064, 0
TBBPA-DBPE	TCI Americas	95	6.4, 0.64, 0.064, 0.0064, 0.00064, 0
TBPH	Accustandard	98.7	0.64, 0.064, 0064, 0.00064, 6.4E-05, 0

Chemicals with asterisks are metabolites measured in toxicokinetic studies. NP, not provided.

(1-chloropropyl) phosphate [TCPP] metabolites), and some of the major hydroxylated PBDE metabolites (Burka *et al.*, 1991; Malmberg *et al.*, 2005; Nomeir *et al.*, 1981). The high MW PBDE decabromodiphenyl ether (BDE-209) was dissolved in acetone prior to diluting in DMSO to prevent it from precipitating out of solution. Standard stock solutions and covered dilution plates were stored at −20°C. All solvents used were high-performance liquid chromatography grade.

Fish husbandry. Wild-type zebrafish (Tropical 5D) were maintained under a 14:10 h light/dark photoperiod at the Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University (Corvallis, Oregon) at densities of approximately 500 fish/50 gal tank in recirculating filtered water (carbon, reverse osmosis) maintained at 28°C and supplemented with salts (Instant Ocean). Spawning funnels were placed in tanks the evening prior to chemical exposures, and the next morning after spawning, embryos were collected, staged, and maintained in sterile incubation dishes under the same conditions as adults (Kimmel *et al.*, 1995). Adult care and breeding were conducted in accordance with protocols under Oregon State University's Institutional Animal Care and Use Committee.

Chemical exposures. At 5 hpf, embryos were enzymatically dechorionated using pronase (90 µl at 25.3 U/µl; Roche, Indianapolis, Indiana) and an automated mechanical dechorionator developed at SARL (Mandrell *et al.*, 2012). Dechorionation procedures followed those outlined in Mandrell *et al.* (2012). Dechorionated embryos (1 embryo/well) were placed in polystyrene 96-well plates (BD Falcon, Corning, Lowell, Massachusetts) containing 90 µl of E2 embryo media using SARL's automated embryo placement system. Embryos were visually inspected under a light microscope after dechorionation and robotic plating to ensure embryo viability and proper staging. For chemical exposures, 2 dilution plates were made per chemical. Plate 1 contained serially diluted chemical in 100% DMSO. Plate 2 was prepared from Plate 1 and contained chemical in E2 embryo media at 10-fold higher concentrations (eg, 0–640 µM at 6.4% DMSO) than the final concentration. Ten microliters of this second dilution plate was spiked into two 90 µl exposure plates containing embryos (eg, final concentration = 0–64 µM at 0.64% DMSO; $n = 32$). Embryos were exposed continuously to flame retardants from 6 to 120 hpf. For some of the flame retardants, low aqueous solubility or limitations in stock concentrations available required that the concentration range to be shifted downward.

Developmental malformation evaluations. At 24 hpf, embryos were evaluated for survival, delays in developmental progression, notochord deformities, and altered spontaneous movements. Embryos that did not move (no body flexions, tail contractions) after 60 s were scored as having altered spontaneous movements. At 120 hpf, larvae were evaluated for survival (MORT) and 17 developmental malformations, including yolk sac edema (YSE) and pericardial edema (PE); body axis (AXIS), trunk length (TRUN), caudal fin (CFIN), pectoral fin (PFIN), pigmentation (PIG), and somite (SOMI) deformities; eye (EYE), snout (SNOU), jaw (JAW), and otolith (OTIC) malformations; gross brain development (BRAIN); notochord (NC) and circulatory (CIRC) deformities; swim bladder presence and inflation (SWIM); and touch-responses (TR). For the TR endpoint, larvae were touched with a probe on the head, body, and tail to test for normal rapid swimming and touch-escape responses. Fish that did not move were scored as having impaired TRs even if they showed no other overt malformations. Binary responses were recorded as either absent (0) or present (1) for each endpoint. Data collection was undertaken using a custom barcoding and tracking system (Zebrafish Acquisition and Analysis Program) to facilitate reliable management of the large amounts of data collected. Statistical analyses were performed using R code with testing methodologies used by Truong *et al.* (2014) to evaluate developmental toxicity of chemicals under the ToxCast program (RCoreTeam, 2014; Truong *et al.*, 2014). Briefly, a binomial test was performed that calculated lowest effect levels (LELs) for each endpoint to identify incidences that exceeded a significant threshold above controls. This test was preferable to a logistic regression as it accounted for the observed nonmonotonicity of flame retardant toxicity.

Embryonic PMRs. PMRs in 24 hpf embryonic zebrafish exposed to flame retardants were measured using a custom built PMR assay tool (PRAT). Starting at approximately 17–19 hpf, embryonic zebrafish begin to spontaneously contract their tails in a reflexive manner with advancing development of sensorymotor neuron interactions and muscle enervations (Kimmel *et al.*, 1995; Kokel and Peterson, 2011; Kokel *et al.*, 2010). This response in embryonic zebrafish has been shown to be highly sensitive to light through nonocular photoreceptors located in the hindbrain (Kokel *et al.*, 2013). The PRAT platform uses PMR light from 2 white L300 Linear Lights (Smart Vision Lights, Muskegon, Michigan) and a high resolution Prosilica GX3300 camera (Allied Vision, Stradtroda, Germany) that is mounted under a 96-well plate holder and coupled to a near-infrared filter to remove image and light distortions. The light cycle consists of following: 30 s period of darkness (Background); pulse of PMR light (Excitation 1); 9 s of darkness; second light pulse (Excitation 2); and 10 s of darkness (Refractory). Within approximately 2 s of the initial pulse of light, embryonic fish will undergo vigorous, high frequency body flexions and tail oscillations. Embryos fail to respond to the second PMR light pulse as basal responses of the neuronal circuitry are nonresponsive or suppressed. Digital video recordings of 17 frames per sec captured 850 frames through the light cycle.

Video analyses were conducted using a custom Matlab program (Mathworks, Natick, Massachusetts) that calculated an index of movement based on pixel differences across each video frame stamp. The Matlab output was further processed and analyzed using custom scripts developed in R language (RCoreTeam, 2014). Specifically, overall patterns of activity within each cycle interval (ie, baseline, excitation, refractory) were compared with those in vehicle controls by (1) estimating the 50% peak difference from controls in either direction and (2) performing a Kolmogorov–Smirnov test that compared the empirical cumulative distribution function between chemical treatments and controls. A Bonferroni-corrected p-value threshold of .01 (0.05/5 treatments = .01) was used to determine statistical significance. Embryos that were dead or malformed at 24 hpf, including those with altered spontaneous movements, were not included. Sample sizes after the removal of dead and malformed animals are provided in the Supplementary material.

Larvae PMR. To further evaluate flame retardant effects on neurological and locomotor behavior, larvae at 120 hpf were subjected to a light–dark PMR assay test using a ViewPoint Zebrabox system and video tracking software (ViewPoint Life Sciences, Lyon, France). Zebrafish larvae display consistent patterns of visual locomotor activity upon alterations between periods of light and dark (Emran *et al.*, 2008; Irons *et al.*, 2010;

Kimmel *et al.*, 1974). When light is applied, larvae slow or stop moving, and when light is removed a pronounced increase in locomotion occurs that gradually subsides as darkness continues. These visual motor response behaviors may be evolutionary-linked adaptive responses to catch prey and avoid predation. For example, evidence suggests that the increased locomotor hyperactivity in response to darkness may be a tractable measure of anxiety (dark avoidance behavior) in zebrafish with decreased activity to continuing darkness proposed to represent habituation (Ali *et al.*, 2011; MacPhail *et al.*, 2009; Rihel *et al.*, 2010; Steenbergen *et al.*, 2011).

The movement of treatment and control larvae was tracked using automated video recordings with a Zebrabox equipped with a 96-well plate holder, internal LED lights for light recordings, and mounted camera. The light–dark cycling consisted of the following: 5-min light acclimation; 5-min dark stimulation; 5-min dark acclimation; 5-min light acclimation. The assay was conducted in the morning of day 5 to help protect against temporal variations. The software tracked short and large distance movements (mm) of larvae every 40 ms and integrated these data in 60 s intervals over the 25-min assay. These integrated data were then further compiled and analyzed using custom R scripts to exclude both dead and malformed larvae, determine total movement (mm; short + large distances) of fish over time, and quantify statistical differences in total motion between treatments and controls (RCoreTeam, 2014). Sample sizes after the removal of dead and malformed animals are provided in the Supplementary material. As larval activity did not meet parametric assumptions of normality, Kruskal–Wallis analyses of variance and Dunn's multiple comparison post tests were used to compare median locomotor activity per minute in treatment versus controls in each of the 5-min light/dark phases. Integrated locomotion measured at each minute was retained as an independent observation to account for the large variation in fish-to-fish movement that is still not well understood in the presence of light/dark stimuli with the PMR assay. Statistical significance was defined at $p < .05$.

Heatmap/hierarchical clustering. A heatmap of flame retardant bioactivity was rendered using the *ggplot2* plotting package for R based on chemical groupings and LELs (Wickham, 2009). LELs were considered optimal for use in this situation because they represented shared values among each of the 3 bioassays and the lowest concentration that elicited a significant effect above background. The chemical groupings were organized based on their dominant structural features. Hence, for example, dechlorane while not an organophosphate was including in the CPE group because it is also a polychlorinated flame retardant. Likewise, non-PBDE BFRs were grouped together even though they have other important structural attributes that could be influencing bioactivity. Hierarchical clustering analyses were conducted using a custom R script (RCoreTeam, 2014). In brief, Euclidean distance-based dissimilarity matrices were computed for each chemical group, and the resulting matrices were clustered using a complete-linkage agglomerative clustering algorithm ("bottom up" approach). With this method, objects were assigned initially to their own clusters with the algorithm proceeding iteratively to join similar clusters until there was just 1 single cluster for each grouping.

Principal component analysis. The R language was implemented with *ggplot2* to construct a principal components analysis (PCA) using a covariance matrix as a dimension reduction tool to find principal components and characterize relationships between individual flame retardants, structures, and toxicity endpoints measured as LELs (RCoreTeam, 2014; Wickham, 2009). Bootstrapped *k*-means clustering algorithms (1000 simulations) were applied to the PCA to find clustering with the highest Jaccard similarity coefficients (cluster 1 = 0.706; cluster 2 = 0.714; cluster 3 = 0.669; cluster 4 = 0.694; cluster 5 = 0.835; cluster 6 = 0.613; cluster 7 = 0.770). Jaccard values that are > 0.75 are considered stable clusters, whereas those > 0.60 suggest clustering patterns. Ellipses drawn for each cluster represent the 95% confidence interval of each cluster center. Flame retardants are identified individually and are colored to denote their chemical class.

RESULTS

Developmental Malformations

Table 4 provides a summary of mortality and developmental malformations observed in 24 and 120 hpf fish exposed to flame retardants. Detailed results for all compounds can be found in the Supplementary material. Of the 44 chemicals targeted, 31 caused significant mortality and morbidity while several elicited no effects, including: DPP, dechlorane, BCPCP, TCPP, MCPP, BDE-3, BDE-183, BDE-209, DE-79 (OctaBDE mixture), 3-OH-BDE-47, TBB, TBPH, and TBBPA-DBPE. In contrast, TBBPA caused the greatest array of teratogenic effects at both 24 and 120 hpf, and mITP was an equally potent toxicant at 120 hpf that also caused multiple defects but was inactive at 24 hpf (Fig. 2). The PBDE congener BDE-100 was one of the more potent flame retardants examined with delayed embryonic development at 6.4E-06 μM exposures that led to high mortality by 120 hpf.

Embryonic PMRs at 24 hpf

Flame retardant effects on PMRs of embryos at 24 hpf are summarized in Table 5 and are depicted as significant hyperactive (↑) or hypoactive (↓) body and tail contractions relative to controls. Detailed PRAT results for each flame retardant are also provided in the Supplementary material. Data are only shown for the initial baseline phase (darkness) and first excitation phase (rapid pulse of light) as these are the only phases in which altered activity was observed in this study. This low baseline activity in the second excitation and refractory intervals helps to further validate the assay as basal activity has been shown to be suppressed in embryos after an initial pulse of light. Compared to controls, 12/16 (75%) of the APE-based flame retardants significantly altered locomotor behavior at 24 hpf with the dominant response being hypoactivity. Significantly reduced PMRs were also measured among fish exposed to some concentrations of the CPE-based chemicals, including TDCPP, BDCPP, and TCPP. Both bisphenol A bis-(diphenyl phosphate) (BDP) and TCEP were the only OPFRs tested that caused a hyperactive response. For the brominated compounds, hypoactive responses also dominated with DE-71 (PentaBDE mixture), BDE-15, BDE-99, BDE-153, and BDE-154, as well as HBCD and TBPH, causing reduced activity compared to controls, whereas BDE-3, BDE-100, and TBBPA exposures resulted in significant hyperactivity. Embryos at the highest concentrations tested (64 μM) were also unable to acclimate normally to baseline conditions of darkness, including those exposed to the APE-based TCP, *o*-TCP, and TPP as well as the PBDE congener BDE-153. Embryos exposed to BDP at the lowest concentration tested (0.00064 μM) were hyperactive during the baseline dark acclimation relative to controls.

TABLE 4. LELs Measured in Embryonic Zebrafish Exposed to Flame Retardant Chemicals

	24 hpf embryos (LEL; μM)		120 hpf larvae (LEL; μM)											
	Mort	DP	Mort	YSE	Ax	E	S	J	PE	PF	CF	T	SB	TR
APE FRs														
BDP	-	-	0.064	-	-	-	-	-	-	-	-	-	-	-
BPDP	-	64	0.064	64	64	64	64	64	64	64	64	-	-	-
EHDP	-	-	64	-	64	-	-	-	64	-	-	-	-	-
IDDP	64	64	0.064	-	-	-	-	-	-	-	-	-	-	-
IPP-1	-	64	64	64	64	-	-	-	64	64	64	-	-	64
IPP-2	-	-	0.0064	64	64	-	-	-	64	-	-	-	-	64
IPP-3	-	64	0.064	64	64	-	-	-	64	64	64	-	-	-
mITP	-	-	-	0.64	0.64	-	0.64	0.64	0.64	0.64	-	-	0.64	0.64
RDP	-	-	0.64	-	-	-	-	-	-	-	-	-	-	-
TBEP	-	-	6.4	6.4E-04	-	-	-	-	-	-	-	-	-	-
TBP	-	-	6.4E-04	-	-	-	-	-	0.0064	-	-	-	-	-
TCP	0.0064	64	0.0064	64	64	-	-	-	64	-	-	-	-	64
o-TCP	-	-	64	-	-	-	-	-	-	-	-	-	-	-
TEHP	-	-	6.4	-	-	-	-	-	-	-	-	-	-	-
TPP	0.64	-	0.0064	64	-	-	-	-	-	-	-	-	-	-
CPE FRs														
TCEP	-	-	0.0064	-	-	-	-	-	-	-	-	-	-	-
TDCPP	64	64	64	-	-	-	-	-	-	-	64	-	-	-
BCPP*	-	-	-	-	-	-	-	-	-	-	0.064	-	-	-
BDCPP*	0.0064	0.0064	0.0064	-	-	-	-	-	-	-	-	-	-	-
PBDE FRs														
BDE-15	-	-	-	-	64	-	-	-	-	-	-	-	-	-
BDE-47	0.064	-	0.0064	-	64	-	-	-	-	-	-	-	-	-
BDE-99	6.4	-	0.064	-	-	-	-	-	-	-	-	-	-	-
BDE-100	-	6.40E-06	6.40E-06	-	-	-	-	-	-	-	-	-	-	-
BDE-153	-	-	0.0064	-	-	-	-	-	-	-	-	-	-	-
BDE-154	-	-	0.0064	-	-	-	-	-	-	-	-	-	-	-
DE-71	0.064	-	0.064	-	64	-	-	-	-	-	-	-	-	-
2,4,6-TBP*	-	-	6.40E-04	-	-	-	-	-	0.0064	-	-	-	-	-
5-OH-BDE-47*	-	-	-	-	-	-	-	-	-	-	6.40E-04	-	-	-
6-OH-BDE-47*	-	-	-	-	-	-	-	-	0.0064	-	-	-	-	-
Other brominated FRs														
HBCD	-	-	-	-	6.4	-	-	-	-	-	-	-	-	-
TBBPA	64	6.4	6.4	-	6.4	-	6.4	0.64	-	-	6.4	6.4	-	6.4

Only flame retardants with adverse effects are reported. Raw data for all the flame retardants are provided in the Supplementary material.
Mort, mortality; DP, delayed progression; Ax, axis; E, eyes; S, snout; J, jaw; PF, pectoral fin; CF, caudal fin; T, trunk; SB, swim bladder. *Metabolites.

Larval PMRs at 120 hpf

Table 6 summarizes flame retardant effects on larval zebrafish photomotor behavior at 120 hpf as significant hyperactive (↑) or hypoactive (↓) movement based on integrated locomotor activity in each of the light/dark phases relative to controls. Additional time series and boxplot data for individual chemicals are provided in the Supplementary material. For the OPFRs, compared to controls, exposure to BDP, *t*-butylphenyl diphenyl phosphate (BPDP), IPP, mITP, resorcinol A bis-(diphenyl phosphate) (RDP), tris (2-butoxyethyl) phosphate (TBEP), TPP, TCEP, TCPP, and TDCPP, as well as some metabolites (BCPP, BDCPP, and MCPP), caused significant hypoactivity under dark stimulation. This hypoactivity extended into the dark acclimation phase for IPP, RDP, TBP, TPP, BCPP, TCEP, TCPP, and TDCPP. In contrast, hyperactive responses were measured for several OPFRs in the dark startle phase (TCP, tris (2-ethylhexyl) phosphate [TEHP], and dechlorane) and dark acclimation phase (BPDP, 2-ethylhexyl diphenyl phosphate [EHDP], TEHP, and BCPCP). TDCPP, BCPCP, TCP, IPP, and BPDP also elicited potent hyperactivity compared with controls during the initial or both light acclimations. Fish were less sensitive to the polychlorinated Dechlorane Plus compound than the CPE-based flame retardants. The PentaBDE commercial mixture DE-71 and PBDE metabolites 6-OH-BDE-47 and 5-OH-BDE-47 caused significant hyperactivity in larvae subjected to dark stimulation, which for DE-71 and 5-OH-BDE-47 was preceded by hyperactivity in both the initial light and dark acclimation phases. For the other PBDE congeners as well as the PBDE metabolites 3-OH-BDE-47 and 2,4,6-TBP, depressed locomotor activity was detected in the dark startle phase as well as in some cases during light and dark acclimations (3-OH-BDE-47, BDE-47, BDE-99, BDE-100, BDE-154, and BDE-209). In addition, compared to controls, TBBPA caused significant reductions in movement during both the dark stimulatory and acclimation phases. Fish that had been exposed to TBB and TBPH were unable to acclimate to light. This inability to acclimate was also observed in the dark acclimation phase among fish exposed to TBPH and HBCD. No significant effects on behavior were observed in larvae exposed to BDE-153, BDE-183, DE-79, or TBBPA-DBPE.

DISCUSSION

The vast majority of flame retardants and metabolites tested in this study were bioactive with most (93%; 41/44) causing

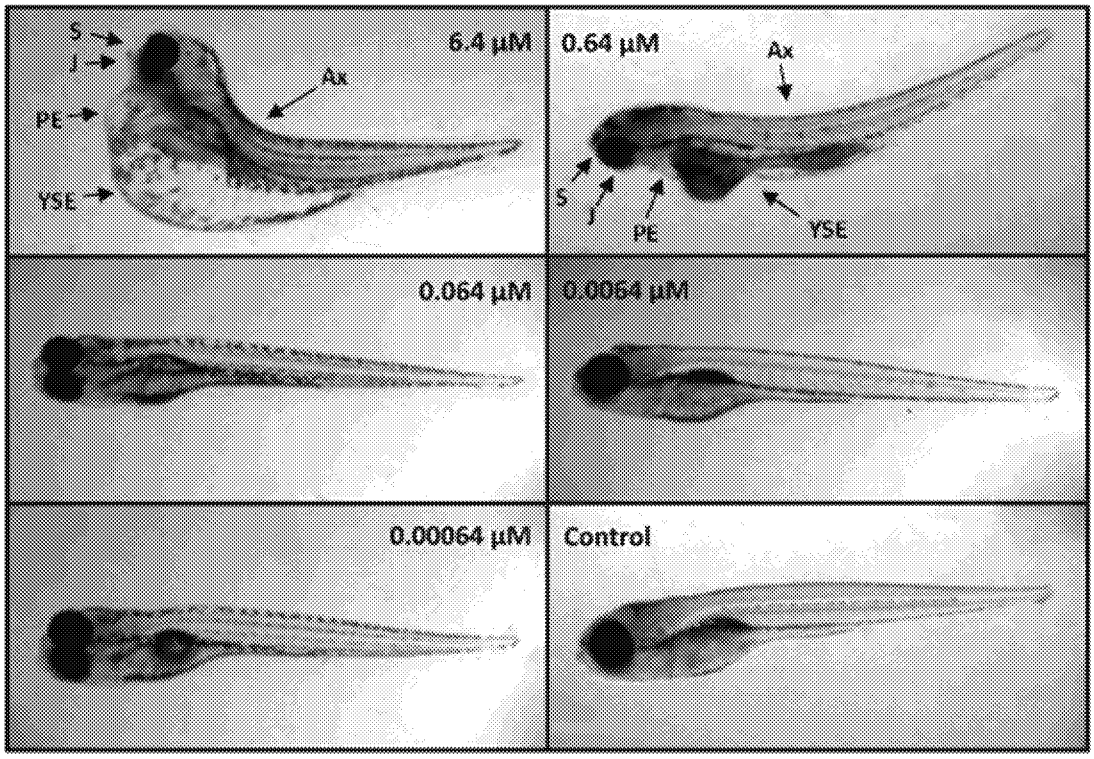


FIG 2. Morphological deformities observed in zebrafish larvae exposed to mITP flame retardant at 0, 0.00064, 0.0064, 0.064, 0.64, and 6.4 µM for 5 days. Deformities denoted as: Ax, axis; S, snout; J, jaw; PE, pericardial edema; YSE, yolk sac edema.

disrupted development in one or more of the 3 bioassays and across one or more concentrations tested (Fig. 3). These findings are noteworthy because the health consequences of exposures to many of these compounds, particularly the APE- and CPE-based chemicals, are poorly understood. The APE-based constituent of FM550 mITP and the brominated-BPA derivative TBBPA elicited the greatest number and variety of developmental malformations, including YSEs, PEs, impaired TRs, and deformities of the trunk, body axis, snout, jaw, caudal fin, and pectoral fins. These results are consistent with studies in rodent models and other research showing that a central mechanism of TBBPA and HBCD developmental toxicity may proceed through disruption of thyroid homeostasis (Eriksson *et al.*, 2006; Kitamura *et al.*, 2002; Mariussen and Fonnum, 2003). The importance of thyroid hormone in brain and somatic development is well established, and small changes in maternal or fetal thyroid hormone can cause severe deformities, motor skill deficiencies, and cognitive impairments (Haddow *et al.*, 1999). In contrast to TBBPA, TBBPA-DBPE was inactive in all 3 bioassays. Additional toxicokinetic studies would be useful to understand TBBPA-DBPE metabolic and elimination profiles as little is known about the toxicity of this chemical. In addition, no effects were measured among embryos exposed to the OctaBDE mixture DE-79 and one of its major components BDE-183. However, the other PBDE parent and metabolites tested were bioactive in one or more of the bioassays with BDE-100 being one of the most potent toxicants tested. The potential for PBDE-induced neurodevelopmental toxicity and thyroid dysfunction are important toxicological endpoints of concern and the data findings here echo these concerns. A number of recent reviews have been written that describe PBDE-induced developmental toxicity and current knowledge of their mechanisms of action (Costa *et al.*, 2014; Noyes and Stapleton, 2014; Staskal and Birnbaum, 2011).

Aryl and Chlorinated Phosphate Esters

All the APE- and CPE-based flame retardants altered 120 hpf larval locomotor behavior at one or more of the concentrations and light/dark epochs examined (Table 6), whereas 75% of the APEs impaired spontaneous motor functioning of embryos at 24 hpf (Table 5). The dominant 24 hpf PRAT response in APE-exposed embryos was hypoactivity that was also detected at baseline for some of these formulations (IPP-2, IPP-3, TCP, o-TCP, and TPP), suggesting that early development prior to 24 hpf may be an important period of heightened susceptibility to this class of flame retardants. The CPEs, in some contrast, were less bioactive at 24 hpf than the APEs, but all impaired behavior by 120 hpf suggesting potentially important target windows for the CPEs after embryonic gastrulation and segmentation. An exception to this finding for the CPEs related to TDCPP and its major metabolite BDCPP, both of which impaired development and depressed movement in 24 hpf embryos. Indeed, this is the first toxicity testing with BDCPP and results here suggest metabolic bioactivation. BDCPP was a substantially more potent teratogen by 4 orders of magnitude (LELs = 0.0064 µM) than TDCPP (LEL = 64 µM), causing reduced survival and impaired development at 24 hpf that led to high mortality by 120 hpf. Moreover, embryonic spontaneous movement at 24 hpf was also significantly depressed among 0.0064 µM concentration groups, suggesting that BDCPP could be an important driver of TDCPP developmental toxicity observed here and in other studies (Dishaw *et al.*, 2011; Fu *et al.*, 2013; McGee *et al.*, 2012). By 120 hpf, both TDCPP and BDCPP elicited similar depressed locomotor phenotypes in fish under dark stimulation, although

TABLE 5. Hyperactivity (↑) and Hypoactivity (↓) Measured as LELs in 24 hpf Zebrafish Exposed to Flame Retardants

	Baseline								Excitation 1							
Conc (μM)	6.40E-06	6.40E-05	6.40E-04	0.0064	0.064	0.64	6.4	64	6.40E-06	6.40E-05	6.40E-04	0.0064	0.064	0.64	6.4	64
APE FRs																
BDP			↑	-	-	-	-				-	-	-	↑	-	
BPDP				-	-	-	-	↓				-	-	↓	↓	↓
EHDP				-	-	-	-	-				-	-	-	-	↓
IDDP				-	-	-	-	-				-	-	-	↓	↓
IPP-1				-	-	-	-	↓				-	-	-	↓	↓
IPP-2				-	-		-	↓				-	-	↓	↓	↓
IPP-3				-	-	-	-	↓				-	-	-	↓	↓
mITP		-	-	-	-	-				-	-	-	-	-		
RDP			-	-	-	-	-					-	-	-	↓	
TBP			-	-	-	-	-					-	-	-	-	
TBEP			-	-	-	-	-					-	-	-	-	
TCP				-	-	-	-	↓				-	-	-	-	↓
o-TCP				-	-	-	-	↓				-	-	-	-	↓
TEHP			-	-	-	-	-				-	-	-	↓	-	
TPP				-	-	-	-	↓				-	-	↓	↓	↓
DPP*			-	-	-	-	-				-	-	-	-	-	
CPE FRs																
BCPCP*				-	-	-	-	-				-	-	-	-	-
BCPP*				-	-	-	-	-				-	-	-	-	-
BDCPP*				-	-	-	-	-				↓	-	-	-	-
Dechlorane			-	-	-	-	-				-	-	-	-	-	
MCPP*				-	-	-	-	-				-	-	-	-	-
TCEP				-	-	-	-	-				↑	↑	-	-	-
TCPP				-	-	-	-	-				-	-	-	-	-
TDCPP				-	-	-	-	-				-	-	-	-	↓
PBDE FRs																
DE-71				-	-	-	-	-				-	-	↓	-	-
DE-79				-	-	-	-	-				-	-	-	-	-
BDE-3				-	-	-	-	-				-	-	↑	-	↑
BDE-15				-	-	-	-	-				-	↓	↓	↓	↓
BDE-47				-	-	-	-	-				-	-	-	-	-
BDE-99				-	-	-	-	-				-	-	-	↓	-
BDE-100	-	-	-	-					-	-	-	↑				
BDE-153				-	-	-	-	↓				-	↓	-	↓	↓
BDE-154	-	-	-	-					-	-	-	↓				
BDE-183	-	-	-	-					-	-	-	-				
BDE-209				-	-	-	-	-				-	-	-	-	-
2,4,6-TBP*			-	-	-	-	-				-	-	-	-	↓	
3-OH-BDE-47*		-	-	-	-	-				-	-	-	↓	-		
5-OH-BDE-47*		-	-	-	-	-				-	-	-	-	-		
6-OH-BDE-47*	-	-	-	-	-				-	-	-	-	-			
Other brominated FRs																
HBCD			-	-	-	-	-					-	-	-	↓	
TBB			-	-	-	-	-					-	-	-	-	
TBBPA				-	-	-	↓	#				-	-	↑	↓	#
TBBPA-DBPE			-	-	-	-	-					-	-	-	-	
TBPH		-	-	-	-	↓				-	-	-	-	-	-	

Dashes (-) indicate no effects compared with controls. Hash tags (#) represent concentrations that caused 100% mortality/morbidity and so were not included. Asterisks (*) denote metabolites.

maladaptation in TDCPP-exposed fish was measured across all 4 light/dark epochs. This difference may be partly attributable to the higher mortality and teratogenicity observed among fish exposed to BDCPP.

The APE-based mITP component of FM550, which has been an important replacement for PentaBDE, was also highly bioactive with results that are consistent with recent studies showing PE and heart malformations (Gerlach *et al.*, 2014; McGee *et al.*, 2013). In this study, no effects were observed in 24 hpf zebrafish exposed to mITP, whereas by 120 hpf, low concentration mITP-exposed larvae presented with multiple morphological and behavioral abnormalities, suggesting that its impacts on neurodevelopment may extend across multiple early life stages with important targets later in development that have yet to be fully described. As for effects of other major components of FM550, TPP also reduced survival with high concentration edemas, and

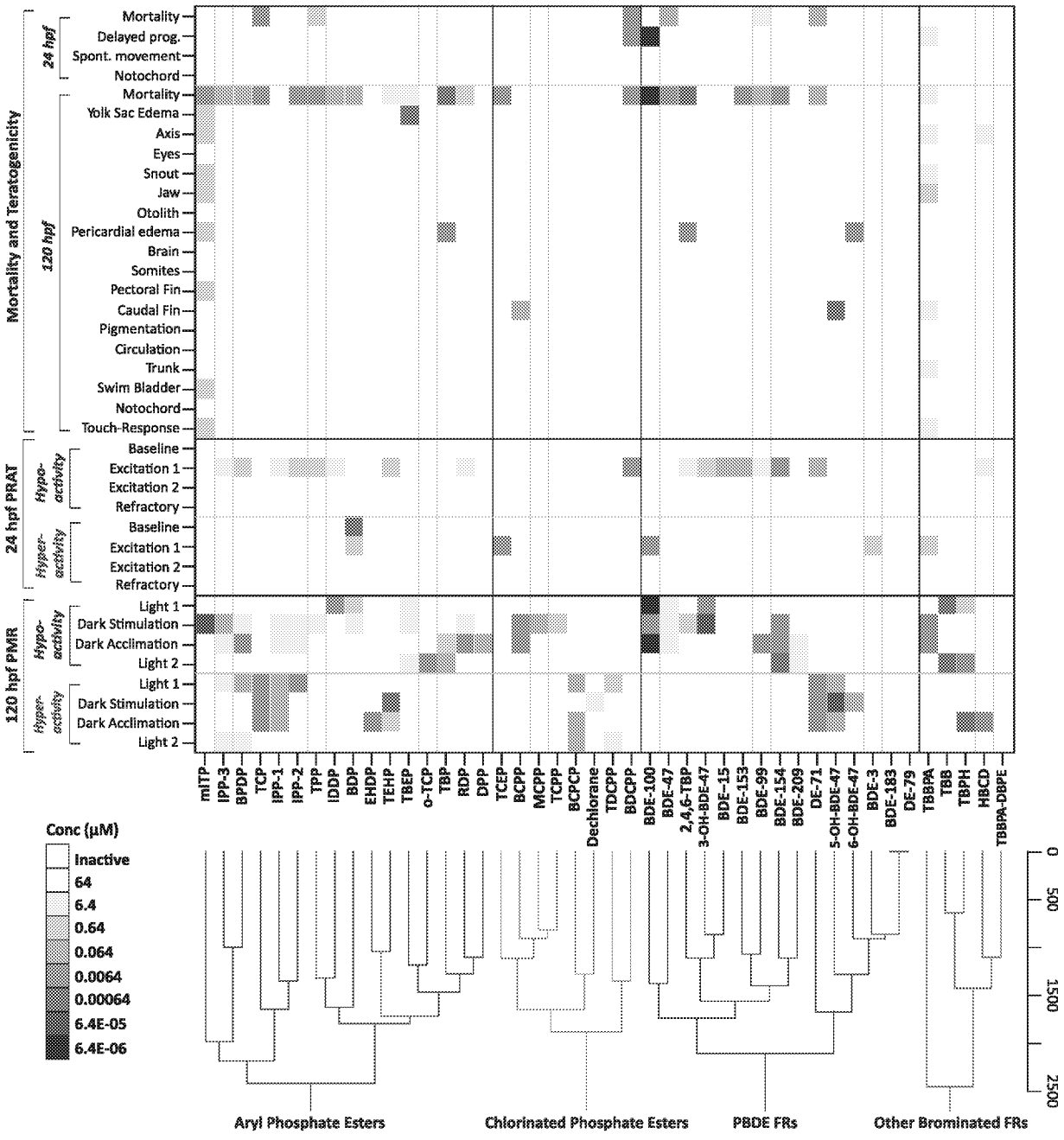


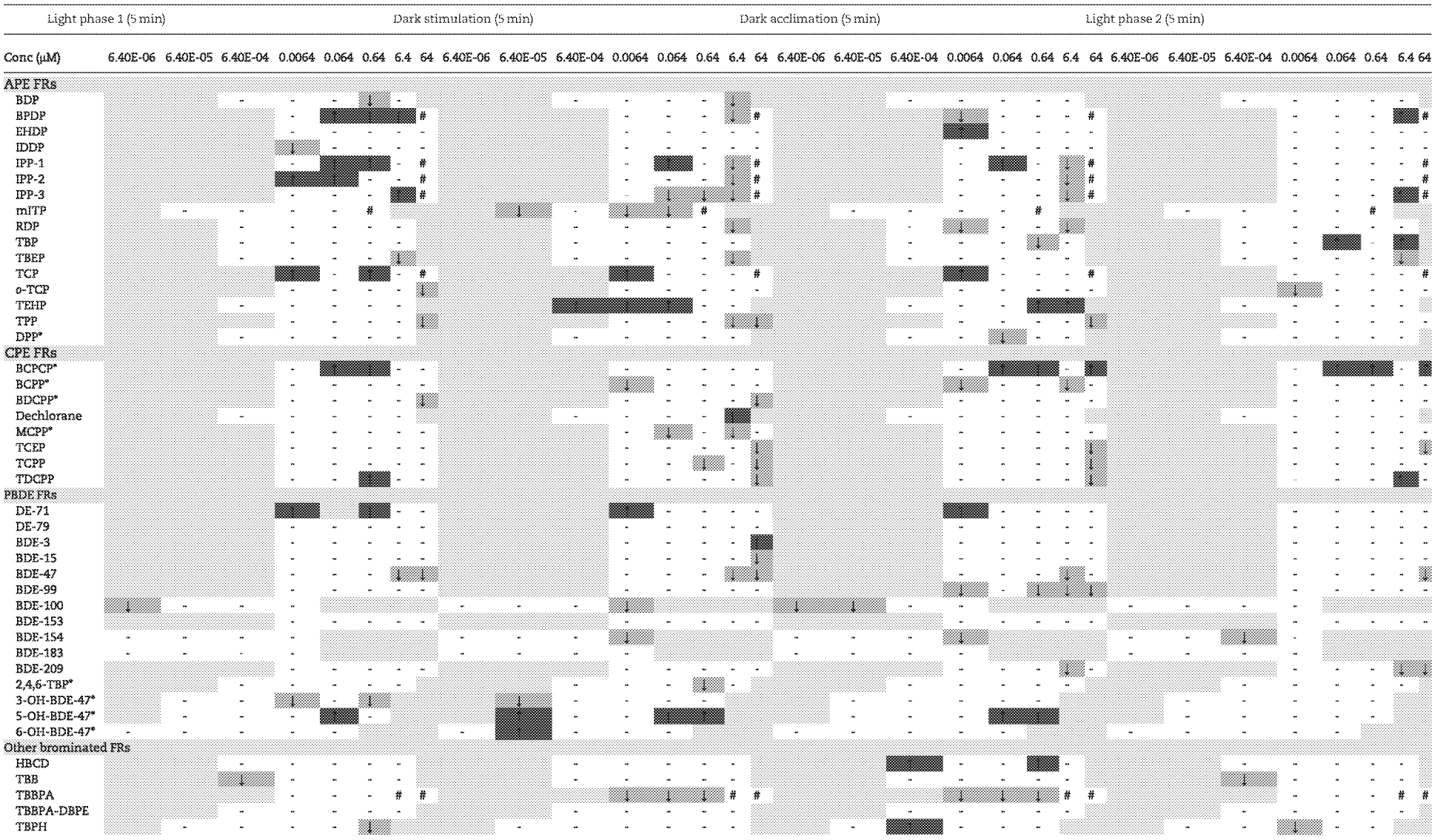
FIG 3. Heatmap and hierarchical clustering of morphological and behavioral responses of zebrafish embryos exposed to halogenated and APE flame retardants. Each square represents the LEL with response profiles hierarchically clustered to link flame retardant structures to bioactivity within chemical groupings.

caused hypoactive locomotor responses at both 24 and 120 hpf, whereas TBB and TBPH caused no significant morphological defects (Tables 4–6, Fig. 3, and Supplementary material). However, compared to controls 120 hpf larvae exposed to TBB and TBPH were unable to acclimate to either light or dark stimuli, and this inability to acclimate was observed earlier in 24 hpf embryos exposed to TBPH. Results here with TPP contrast older screens in adult rodents that generally indicated a lack of neurotoxicity (Sobotka *et al.*, 1986). However, this is one of only a couple developmental neurotoxicity examinations of TPP (McGee *et al.*, 2013) and data suggest that younger animals may be more susceptible to this chemical thereby warranting further study.

Common Responses/Mechanisms of Bioactivity

One of the ongoing challenges with characterizing toxicity results from larger chemical data sets such as this flame retardant grouping relates to effectively visualizing and dissecting potentially related responses and common toxicity mechanisms among a high-dimensional, complex set of morphological and behavioral phenotypes. For this study, 2 approaches were adopted to examine relationships of bioactivity within and across individual chemical classes. The first approach (Fig. 3) used a heatmap and hierarchical cluster analysis to evaluate interactions and differences in bioactivity within chemical groupings based on LELs. The second approach used a PCA test as a dimension reduction tool to further address whether and

TABLE 6. Hyperactivity (↑) and Hypoactivity (↓) Measured as LELs in 120 hpf Zebrafish Exposed to Flame Retardants



Dashes (-) indicate no effects compared with controls, hash tags (#) represent concentrations that caused complete or near 100% mortality/morbidity, and asterisks (*) denote metabolites.

how compounds clustered based on their structures, teratogenicity, and neurobehavioral activity. Figure 4 shows a 2-dimensional PCA of the first 2 principal components. Loadings for each component are provided in the Supplementary material.

While all the APE-based chemicals tested had effects on development, those APEs with isopropyl (ie, mITP, IPP), butyl (BPDP), and cresyl (TCP) substituents on phenyl rings clustered tightly and were particularly potent across the 3 bioassays (Fig. 3). This clustering pattern suggests that these types of structural moieties and substitution patterns on phenyl may enhance the overall biological reactivity of this chemical class. As for the CPEs, TDCPP and BDCPP clustered tightly and may be indicative of the greater chlorination of TDCPP and BDCPP as well as hydroxylation of BDCPP that could be influencing their bioactivity and earlier target window at 24 hpf. The other chlorinated-tris compounds characterized, TCPP and TCEP, also clustered, with the TCPP metabolites BCPP and MCPP clustering with TCPP suggesting similar reactive features. In contrast, TCEP and its metabolite BCPCP did not cluster tightly, as BCPCP elicited potent hyperactivity in both the light and dark acclimation phases of the larval PMR assay, whereas TCEP was bioactive across several endpoints. PBDE clustering appeared to be based on patterns of bromination with congeners having between 2 and 6 bromines generally demonstrating the greatest bioactivity. BDE-100 and BDE-47 clustered tightly based on comparatively potent teratogenicity and hypoactivity in the larval PMR assay regardless of whether light or dark stimulation was applied. These compounds are structurally identical except that BDE-100 contains an additional ortho-substituted bromine atom on diphenyl ether. Indeed, a number of parent PBDEs (BDE-47, -15, -153, -99, -154, -209) and metabolites (2,4,6-TBP, 3-OH-BDE-47) clustered due to hypoactivity in one or both PMR assays and elevated mortality/morbidity. In contrast, PentaBDE (DE-71), which is composed of some of these parent PBDEs (BDE-47, -99, -153, -154), elicited a hyperactive response suggesting that zebrafish are highly sensitive to other components of DE-71.

Results of the 2-dimensional PCA (Fig. 4) and k-means derived clusters support that flame retardant bioactivity was not driven by major structural groupings, which was somewhat expected as nearly all (41/44) of the flame retardants and metabolites tested were deleterious in one or more of the bioassays across multiple endpoints and concentrations. Nonetheless, it is possible from the pattern of clustering in the PCA to discern groupings with levels of reduced and enhanced activity. Cluster 5 is notable because it includes several compounds, all brominated except for dechlorane, that produced little to no effect. The exception to this group 5 clustering was the PBDE metabolites 5-OH-BDE-47 and 6-OH-BDE-47 that caused low concentration hyperactivity in the 120 hpf PMR assay along with low concentration caudal fin and PEs, respectively, but elicited no effects in the 24 hpf-PRAT assay. These metabolites appeared to group with this cluster of comparatively inactive compounds because the 120 hpf-PMR data did not contribute substantially to the principal components, whereas the PRAT data were important to the loadings (see Supplementary material for PCA loadings).

At the other extreme, cluster 4, which is widely separated from cluster 5, contained only mITP and TBBPA as these were the most developmentally toxic flame retardants measured in this study. Clusters 3, 6, and 7 were notable because they contained generally the next most highly bioactive flame retardants after cluster 4. PentaBDE (DE-71) and all of its major components (BDE-47, -99, -100, -153, and -154) clustered in one of these 3 groupings along with one of its major hydroxy metabolites 2,4,6-TBP. This is concerning as these congeners continue

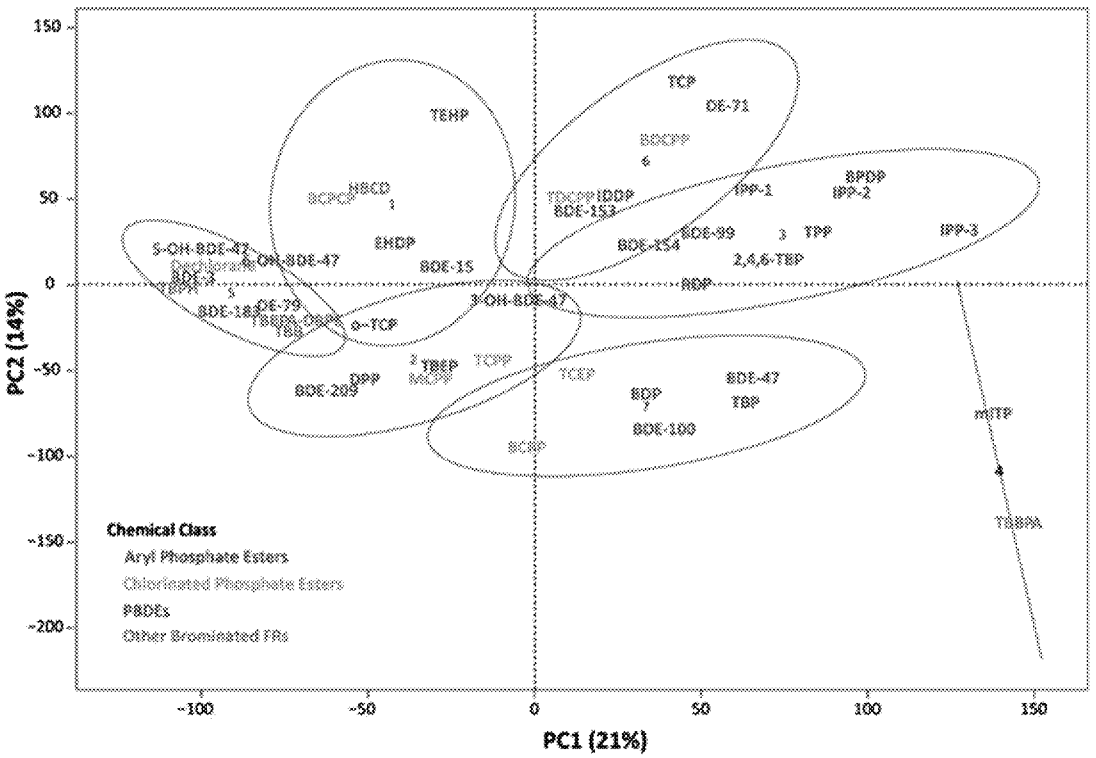


FIG 4. Two-dimensional PCA and covariance matrix identifying regional clustering patterns among flame retardants based on their developmental and behavioral toxicity measured as LELs in 24 and 120 hpf zebrafish exposed to chemical from 6 to 120 hpf.

to be highly detected PBDEs in humans and the environment (Toms *et al.*, 2011). It is also notable that TPP, which along with mITP are major components of the PentaBDE replacement FM550, also clustered in group 3 along with the 3 bioactive IPP formulations, RDP, and BPDP. This is contrasted by the other major constituents of FM550, TBB and TBPH, that clustered with other less bioactive flame retardants in group 5. As for the CPEs and their metabolites, both TDCPP and its metabolite BDCPP clustered in group 6 further supporting the potential importance of BDCPP in the toxicity of TDCPP. Similarly, BCPP appeared to be a more potent bioactive metabolite than its parent TCPP.

Concordance of Bioassay Results

There was generally a high degree of concordance among the 3 assays in that a "hit" or lack thereof in 1 assay was typically predictive of the presence and absence of effects in another assay with some exceptions (Fig. 5). Both the 24 and 120 hpf PMR assays predicted the presence and absence of morphological defects at 120 hpf for 93% (41/44) of flame retardants tested. The exceptions to these interactions were for TCPP, 3-OH-BDE-47, and BDE-3 that elicited effects in both behavioral assays but were not morphologically sensitive. These results support the use of these types of PMR assays as promising nonlethal means to efficiently characterize developmental abnormalities in young zebrafish. This attribute may be particularly meaningful for future studies seeking to understand the long-term consequences of exposures to these and other hazardous compounds in older animals.

Another observation pertains to a comparison of potency observed in the morphological and behavioral assays. Figure 3 shows that there were some chemicals that elicited significant behavioral changes at lower concentrations than those concentrations reducing survival or causing other morphological deformities. Conversely, there were also instances where survival and morphology were the most sensitive targets. These differences in relative potencies across the different bioassays could be meaningful to understanding windows of susceptibility and mechanisms of action but could also be linked to conditions of the study design. Although the PMR assays were conducted in animals that appeared to be visually normal and healthy with otherwise unaltered morphology or TRs, it is possible that some of these animals were experiencing systemic toxicity or other unobservable deformities (eg, musculoskeletal impairments) that were not readily discernable with the morphological evaluation. This could have skewed the behavioral assays to identify positive hits if animals were experiencing systemic toxicity that was not detected during the morphological evaluations. It is also possible that our testing conferred some "survivor" bias. That is, for compounds and test concentrations that caused death, it is possible that there could be a subsample of animals being tested in the PMR assays that were more resistant to the chemical and so effects on behavior might not be observed or could be reduced.

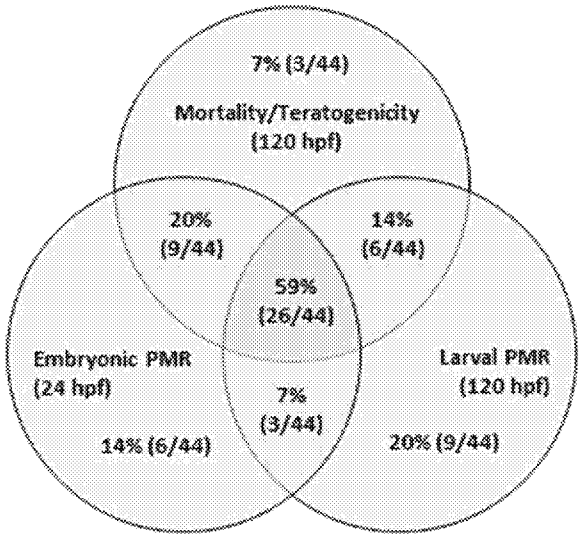


FIG 5. Venn diagram showing distribution and concordance of flame retardant bioactivity and inactivity measured in 3 zebrafish bioassays used to characterize developmental toxicity.

This is the first study to use the 24 hpf PRAT assay to evaluate flame retardant effects at initial stages of the developing zebrafish nervous system. There were several examples of compounds (BDP, EHDP, TEHP, TDCPP, BDE-100, DE-71, TBBPA, and HBCD) whereby directionally opposite behavioral responses were measured at 24 and 120 hpf. For instance, the APE-based flame retardant BDP caused low concentration mortality at 120 hpf in concert with increased and decreased locomotor responses at 24 and 120 hpf, respectively. There were also several instances where chemical sensitivities were detected in the 120 hpf PMR assay but not in the 24 hpf PRAT assay (mITP, TBEP, TBP, DPP, BCPP, MCPP, BCPCP, dechlorane, BDE-47, DE-71, 5-OH-BDE-47, 6-OH-BDE-47, TBB, TBPH), and vice-a-versa (BDE-153). These opposing responses are notable because they could reveal important differences in targets and mechanisms for these compounds. Compelling evidence now supports that 24 hpf PRAT excitatory motor responses in zebrafish are linked to nonocular PMR photoreceptors and distinct neuronal pathways activated in the caudal hindbrain—but not in the forebrain and midbrain—that may involve opsin-based phototransduction pathways (Kokel *et al.*, 2013). Thus, PRAT can reveal important chemical sensitivities at some of the earliest stages of anatomical and functional patterning of the vertebrate nervous system.

While the underlying mechanisms driving larval PMR responses to light and dark and other extrinsic/intrinsic stimuli are still not well understood, it is readily evident that the central and peripheral nervous system of zebrafish is much more complex by 120 hpf than at 24 hpf. With this advancing development, a variety of toxicity mechanisms could be operating that might include chemical-induced changes in biochemical levels/activity, electrical signaling, receptor-mediated functioning, cell–cell communications, and the responsiveness and plasticity of developing organ systems. Another important difference that could be impacting the directionality of behavioral results is that the exposure duration is longer in the 120 hpf fish and so differing patterns of uptake and toxicokinetics in older larvae could elicit different patterns of toxicity and chemical sensitivities. Pairing these PMR assays together at 24 and 120 hpf is advantageous because we are able to derive a more complete picture of flame retardant effects on early development that are homologous to fundamental processes of development in humans (Selderslaghs *et al.*, 2013). For instance, mechanistic data suggest that PBDE neurotoxicity may operate by several pathways that include disrupted thyroid hormone signaling (Ibhazehiebo *et al.*, 2011; Noyes *et al.*, 2013), altered cholinergic neurotransmissions (Dufault *et al.*, 2005; Johansson *et al.*, 2008), impaired neuronal proliferation and plasticity (Ibhazehiebo *et al.*, 2011; Xing *et al.*, 2009), and oxidative stress (Tagliaferri *et al.*, 2010). Furthermore, consistent with results here, a substantial number of human epidemiology studies and

neurodevelopmental toxicity studies in rodents and other models, which have been summarized in recent reviews (Costa *et al.*, 2014; Staskal and Birnbaum, 2011), have shown that PBDEs can elicit adverse neurobehavioral outcomes in early development. Data generated here are consistent with and complement these other studies in humans and laboratory models. However, while PBDE biological disposition and toxicity are increasingly well-described, our understanding of the underlying mechanisms of toxicity for the other BFRs and OPFRs remains limited. Data generated here may be particularly helpful in future research. Moreover, other classes of organophosphates, such as organophosphate pesticides like chlorpyrifos, which have been shown to interfere with neurodevelopment by cholinergic and non-chlorinergic pathways, have been subjected to more testing than the OPFRs and may provide helpful insights into future testing that could complement data generated here and elsewhere (Levin *et al.*, 2004; Yang *et al.*, 2011).

CONCLUSIONS

Taken together, results of this study indicate that zebrafish neurological and morphological development appears to be highly sensitive to many of the flame retardants currently in use and present in humans and wildlife, as well as many being considered and used as replacements. This finding takes on heightened importance because these chemicals are not typically detected in humans and the environment in isolation but are present rather as complex mixtures. This type of high throughput screening methodology in zebrafish provides a meaningful opportunity moving forward to design flame retardants that impart reduced human health and environmental hazard potential. Not only do these assays detect initiating events of neurological impairments but also effects on gross morphological development. These types of readouts allow for the anchoring of developmental toxicity to morphological and behavioral phenotypes that in turn can be used to understand toxicity pathways for ultimate translation to humans. Future work will involve using this *in vivo* testing platform in young zebrafish to identify important mechanistic targets for these compounds and developmental windows of susceptibility. Finally, this research platform is well suited to examine the interactive effects of complex mixtures to discern differential or synergistic developmental toxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Peer Review Publication 2:

Noyes PD, Garcia GR, Tanguay RL. 2016. Zebrafish as an in vivo model for sustainable chemical design. *Green Chemistry* 18:6410-6430.

Basis for inclusion and scientific impact:

An important organizing principle for EPA's chemical safety for sustainability (CSS) strategic research action plan is based on challenges that despite the essentiality of chemistry to modern life, there continues to be a need for innovative, systematic, effective, and efficient approaches and tools to inform decision-making to reduce human health and environmental impacts. This global problem as well-articulated by the agency is what really led me to my fellowship in Dr. Tanguay's laboratory. In addition to my individual and collaborative research, I think an important job of a researcher in human health and environmental toxicology involves positioning their work in a larger context. This paper represents this type of effort to translate and synthesize a great deal of data and tools, including my own, to help advance the field of toxicology. It represented an invited review by the journal Green Chemistry to describe how innovations in the zebrafish model have positioned it as a rapidly advancing model that can be employed to design safer chemicals.

As mentioned, Dr. Tanguay has been on the leading edge world-wide in designing in vivo high-throughput screening (HTS) assays with zebrafish, and conducting and linking these HTS assays and data outputs to other molecular testing (e.g., RNA sequencing, microarray) and neurobehavioral testing platforms. I had the privilege of working with him on these efforts, and conducted a great deal of research in his laboratory, and in collaboration, on all of these fronts to advance the design of methods and tools to use zebrafish as high-throughput in vivo models to understand toxicity pathways and enhance the design of more sustainable chemistries. This particular manuscript in Green Chemistry describes the cutting edge high-throughput molecular screening, including those using RNA sequencing and microarray technologies. It describes in greater detail the uses of fish embryos as HTS biosensors to rapidly screen chemicals for bioactivity, including their tremendous application potential to R&D both inside the government and in the private sector. The high-throughput behavioral assays being developed provide a promising linkage of neurodevelopmental malformations to behavioral responses that are increasingly important to understanding the human health impacts of chemical exposures. Perhaps an even more important impact of this paper is a discussion of how these assays across different platforms and levels of biological organization can be integrated to provide a fuller picture of the molecular targets for chemical interaction and the downstream cascades of events that can culminate in an adverse outcome.

I completed all aspects of this review, including developing the focus and outline, designing and managing the literature survey and data review, drafting all sections, and deriving conclusions. I also thought it was important to describe major limitations and uncertainties in zebrafish HTS assays, and identify research and steps that need to be taken to further their advancement. I also responded to and managed the peer review process.

Green Chemistry

CRITICAL REVIEW

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Zebrafish as an *in vivo* model for sustainable chemical design

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Heightened public awareness about the many thousands of chemicals in use and present as persistent contaminants in the environment has increased the demand for safer chemicals and more rigorous toxicity testing. There is a growing recognition that the use of traditional test models and empirical approaches is impractical for screening for toxicity the many thousands of chemicals in the environment and the hundreds of new chemistries introduced each year. These realities coupled with the green chemistry movement have prompted efforts to implement more predictive-based approaches to evaluate chemical toxicity early in product development. While used for many years in environmental toxicology and biomedicine, zebrafish use has accelerated more recently in genetic toxicology, high throughput screening (HTS), and behavioral testing. This review describes major advances in these testing methods that have positioned the zebrafish as a highly applicable model in chemical safety evaluations and sustainable chemistry efforts. Many toxic responses have been shown to be shared among fish and mammals owing to their generally well-conserved development, cellular networks, and organ systems. These shared responses have been observed for chemicals that impair endocrine functioning, development, and reproduction, as well as those that elicit cardiotoxicity and carcinogenicity, among other diseases. HTS technologies with zebrafish enable screening large chemical libraries for bioactivity that provide opportunities for testing early in product development. A compelling attribute of the zebrafish centers on being able to characterize toxicity mechanisms across multiple levels of biological organization from the genome to receptor interactions and cellular processes leading to phenotypic changes such as developmental malformations. Finally, there is a growing recognition of the links between human and wildlife health and the need for approaches that allow for assessment of real world multi-chemical exposures. The zebrafish is poised to be an important model in bridging these two conventionally separate areas of toxicology and characterizing the biological effects of chemical mixtures that could augment its role in sustainable chemistry.

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Introduction

Humans and wildlife are exposed to an ever-increasing variety of man-made chemicals and complex chemical mixtures. Some of these chemicals have proven to be highly persistent and do not degrade appreciably in humans or the environment, thus presenting long-term exposure concerns and disease susceptibilities. In some instances, they have shown a propensity for long-range transport by the Earth's climate and weather systems being deposited in higher latitudes and some of the most remote places on the planet.[1] In fact, synthetic chemicals are now found in every habitat of the planet and hundreds are detected in a variety of life forms from microbes to plants extending through food webs up to apex predators and humans.[2–5]

Historically, efforts to control chemical releases to the environment have involved technologies and approaches that reduce or clean up releases after the fact. These "end of pipe" strategies, while relevant, are being replaced with advances in pollution prevention technologies that include chemical reagents, processes, and products that are less hazardous and more sustainable. The publication of Paul Anastas and John Warner's important book, Green Chemistry: Theory and Practice, in 1998, has expanded the field of green chemistry significantly in the last 20 years and it is now an established scientific discipline.[6] This book established 12 principles of green chemistry that remain an important organizing framework that guides industry, academic, and government scientists. The pursuit of green chemistry goes beyond waste

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reduction and pollution prevention, and targets opportunities for design innovation over the entire life cycle of materials (*e.g.*, chemical) to minimize effects on humans and the environment.[7] Closely related and integrated with the principles of green chemistry and one of its greatest challenges centers on emphasizing and adopting more sustainable chemistry practices in chemical design.[8–10] This issue is captured succinctly by Collins (2001)[8] in describing man-made chemical design as one that has tended to implement simple reagent designs but by using a vast array of elements. This is contrasted by natural systems that do the opposite, employing just a limited number of environmentally common elements but with a diversity of biochemical processes to select for the desired product or process. With biomimicry in mind, there have been promising efforts to produce more sustainable plastics using zeolites (microporous aluminosilicate minerals) to catalyze the transformation of microbially-synthesized lactic acid to lactide, which is a key precursor to biodegradable polylactic acid plastics that has historically been a costly step in production.[11] Alternative peroxide activating catalysts, tetraamido macrocyclic ligands (TAMLs), have also been designed as oxidation catalysts with a number of structural variants that have shown promise in many uses, including in water disinfection, pulp bleaching, and the break-down of a growing number of persistent chemicals, including chlorinated phenols, explosive residues, dyes, pesticides, and synthetic estrogens.[12–15] These are but just a couple of examples of looking to sustainable chemistry in designing functional yet more benign chemicals. However, while these types of efforts are laudable and hold promise, the widespread integration of green and sustainable chemistry into chemical design remains elusive.[16–19]

In particular, the scale of synthetic chemical production continues to be enormous with thousands of chemicals used today throughout the world as industrial feedstocks, pesticides, pharmaceuticals, and nanoparticles, among many other industrial and household uses. There are currently about 85 000 chemicals that have been produced or imported for sale in the U.S. since chemical inventory tracking was established in 1979 under the Toxic Substances Control Act (TSCA), the primary U.S. statute that oversees industrial chemicals as amended (Frank R. Lautenberg Chemical Safety for the 21st Century Act); however, this does not reflect industrial chemicals currently on the market because it is a cumulative running total. In its most recent data collection, the U.S. EPA reported that roughly 7700 chemicals subject to TSCA chemical data reporting requirements were produced or imported into the U.S. at more than 25 000 pounds (reporting threshold) during 2011.[20] By volume this equated to chemical production or importation volumes of about 9.5 trillion pounds per year or 26 billion pounds per day.[21] This U.S. snapshot becomes even more astonishing as it does not include chemicals that are exempt from TSCA or regulated under other statutory authorities, such as pesticides, drugs, food additives, and tobacco products. For instance, in both 2006 and 2007, approximately 5.2 billion pounds and 1.1 billion pounds of pesticide active ingredient were applied globally and in the U.S., respectively, covering several hundred biologically active agents that are regulated in the U.S. under separate statutory authority.[22] The story is similar in the E.U. where estimates report roughly 100 000 chemicals available for use. Regulations enacted in 2006 under the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) program seek to evaluate the safety of approximately 30 000 of these chemicals.[23] Similar REACH-types of legislation also have been enacted in Asian countries, including China and South Korea, but the extent of chemical usage is not well described in most countries, particularly those that are less economically developed.

Thus, current statutory and regulatory requirements have resulted in a limited number of environmental chemicals, such as pesticides and some industrial chemicals, being subjected to more rigorous testing and safety evaluation prior to market introduction. It also continues to be challenging for government entities to balance protecting confidential business information (CBI) with ensuring the public's right-to-know so that there is adequate transparency surrounding the composition and safety of chemical products. For the remainder of the many thousands of chemicals being used today generally less is known about their toxicity potential in humans and wildlife. This is not to say that there has not been important toxicity testing of non-pesticides, but oftentimes detailed focus by the broader research community occurs after or in response to chemicals being detected in humans and the environment. Moreover, most human health and ecological effects data used in hazard evaluations for chemical risk assessment continue to focus on direct measurements of apical outcomes of concern, such as reproduction and survival. Analyses typically rely on empirical testing of a single chemical in vertebrate models, such as rodents, with application of uncertainty factors to extrapolate toxicity findings across species and exposure concentrations. Increasingly, these traditional approaches of single chemical testing with *in vivo* animals are recognized as impractical as evidenced by the vast resources and time that would be needed to test the enormous backlog of chemicals and environmental mixtures for which less evaluation has occurred and the many new chemistries coming to market each year. Indeed, it has been several years now since the U.S. National Research Council (NRC) recommended that shifts were required in human health toxicity testing from whole animal test systems to *in vitro* methods and bioinformatics to better evaluate biological perturbations and toxicity pathways (Fig. 1).[24] Ecological endpoints have also been targeted. For example, a Society of Environmental Toxicology and Chemistry (SETAC) Pellston workgroup examined how to better incorporate mechanistic data into predictive ecotoxicity testing and risk assessment.[25] There is also increasing interest in developing test methods and alternative techniques that consider animal welfare and minimize the use of animals in pharmaceutical and chemical testing.[26,27] Thus, the field of toxicology has been shifting from traditional testing methods with whole organisms and single chemical analyses to more

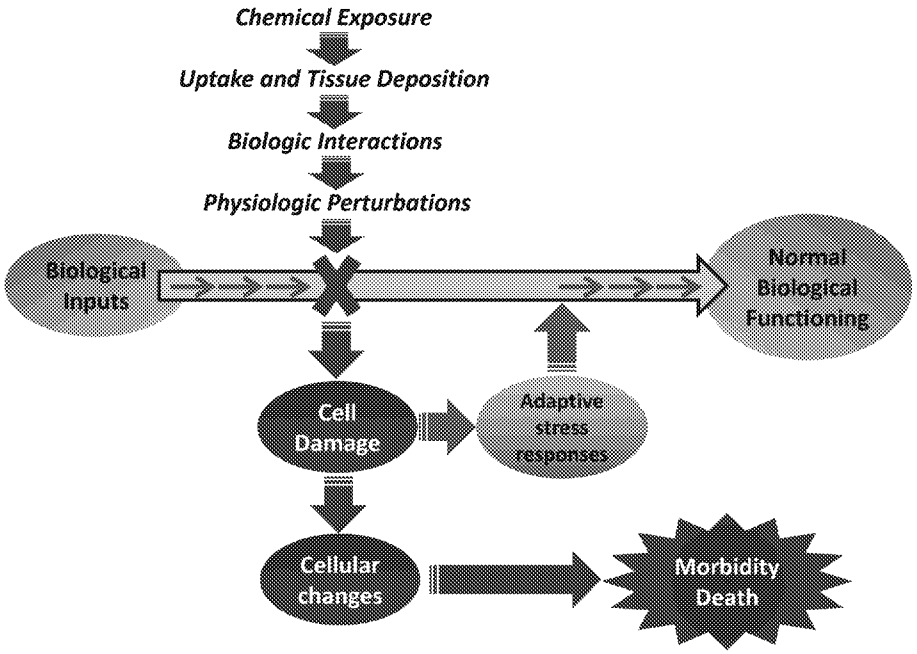

Fig. 1 Toxicity pathways leading to perturbed biological responses with chemical exposures. Depending on the potency of the chemical exposure and other biological factors (*e.g.*, life stage, nutritional status, genetics), humans and wildlife may be unable to adapt to toxicant exposures and normal physiological functioning is compromised leading to disease and/or death. (Adapted from NRC 2007.)

predictive-based approaches that strive to characterize early molecular initiating events and biological modes of action (MOAs) to identify chemical and chemical classes that warrant enhanced scrutiny and testing.

This shift in emphasis toward predictive-based approaches and reductions in animal usage in chemical testing has led to increasing interest in the use of *in vitro* and non-mammalian models, particularly embryonic zebrafish, as biosensors to test for bioactivity potential. To help prioritize chemicals for testing, the U.S. Environmental Protection Agency (EPA), National Institute of Environmental Health Sciences (NIEHS), and Food and Drug Administration (FDA) formed a consortium "Tox21" to apply high-throughput technologies to screen roughly 10 000 chemicals and characterize molecular and biological targets and pathways underlying toxicity (http://www.epa.gov/ncct/Tox21/). In addition, the U.S. EPA launched its ToxCast™ program in 2007 to further develop HTS technologies with cell-based approaches and embryonic zebrafish to screen chemical bioactivity.[28–31] These types of predictive-based test methods with zebrafish provide an opportunity to design and promote inherently safer chemicals and undertake bioactivity evaluations early in the chemical discovery process. The U.S. EPA also has initiated a multi-year transition as part of its Endocrine Disruptor Screening Program (EDSP) toward adoption of HTS and computational toxicology approaches to screen thousands of pesticides and drinking water contaminants for possible endocrine bioactivity in humans and wildlife.[32] Likewise, the OECD has an extensive test guideline program in place to support member country efforts to test chemicals for potential endocrine activity of which zebrafish testing is included.[33] Finally, there have been efforts by experts in green chemistry and environmental health to design and implement frameworks to guide testing early in chemical design (*e.g.*, Tiered Protocol for Endocrine Disruption; TiPED[10]), reduce laboratory animal use (Alternatives to Laboratory Animals; ATLA[34]); and evaluate evidence for endocrine activity (*e.g.*, Systematic Review and Integrated Assessment; SYRINA[35]).

This review describes zebrafish testing strategies that are advancing our ability to design safer chemicals. It discusses the rapidly expanding use of the zebrafish model in genetic toxicology, neurobehavioral testing, and as a core *in vivo* model in the fields of HTS and computational testing, and how these technologies can support sustainable chemistry and be positioned to inform early chemical design. While not without challenge, these technologies are contributing to a rich array of more efficient tools to characterize chemical bioactivity and toxicity pathways across multiple levels of biological organization (Fig. 1 and 2). We consider advances in zebrafish testing strategies that are not only expanding our understanding of chemical effects on human health but also among natural biota in ecotoxicology. As such, there is discussion of efforts to use zebrafish as an integrative model in wildlife toxicity screening and in characterizing chemical effects on endocrine system functioning. One of the clear strengths of the zebrafish model is the utility it confers in being able to evaluate chemical effects across different levels of biological organization. The popularity of the zebrafish as a vertebrate model of human disease and chemical toxicity relates to the balance it confers in providing meaningful biological complexity but

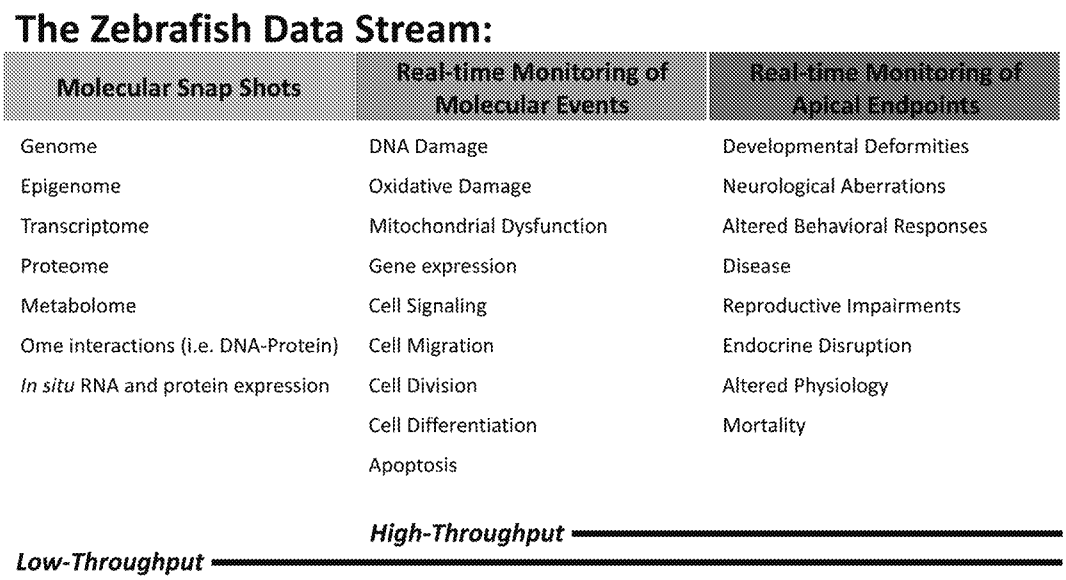

Fig. 2 Conceptual diagram of the zebrafish data stream across multiple levels of biological organization.

practical utility as they can be modified genetically and pharmacologically thereby filling an important niche between invertebrate models, such as fruit flies and worms, and more costly mammalian models. Approximately 70% of protein-coding genes and over 80% of disease-related morbidity genes have been shown to have at least one ortholog in zebrafish, making them a genetically tractable vertebrate model to humans.[36]

The zebrafish also has generally well-conserved organ systems, tissues, and cell types that make it informative to studying vertebrate development (Table 1). This high degree of conservation makes zebrafish highly applicable to examining chemical effects on embryogenesis that is translational to humans and other vertebrate taxa.[37] Moreover, while *in vitro* test systems and 'omic' technologies (*e.g.*, genomics) confer many advantages and hold great promise, it can be challenging to reproduce results in animal models as biological complexity, such as toxicokinetics and organ system plasticity, are not easily predicted. Similarly, persistent issues remain in interpreting changes in expression (*e.g.*, transcript levels) as being adaptive or toxic with current *in vitro* systems. Thus, chemicals that elicit activity in *in vitro* assays may require testing and validation with *in vivo* models. Finally, ongoing pressures to reduce the large numbers of animals used in chemical discovery and safety testing have prompted focus on alternative models and zebrafish offer a biologically relevant choice.

Table 1 Comparison of early developmental of human, rat, and zebrafish[37,255–257]

Developmental stage	Human (day)	Rat (day)	Zebrafish (hour)
Blastula/blastocyst	4–6	3–5	2–5
Implantation	8–10	6	n/a
Neural plate formation	17–19	9.5	10
First somite	19–21	9–10	10–11
10 somite stage	22–23	10–11	14
Neural tube formation	22–30	9–12	18–19
First pharyngeal arch	22–23	10	24
Initiation of organogenesis	21	5	10
First heartbeat	22	10.2	24
birth/hatching	253	21	48–72

Genetic toxicology and safer chemical design

The use of embryonic zebrafish screens in chemical testing had its genesis in developmental biology with efforts to clarify genes involved in vertebrate development. Some of this work employed small molecules as chemical probes to alter gene functions and products to induce non-heritable phenotypes that could in turn help reveal early developmental processes.[38] The rapid pace of advances in genetic screening provides an opportunity to integrate molecular endpoints into safer chemical design and to foster evaluation of the many thousands of chemicals already in use. Methods that allow for control of gene expression can be used to characterize toxic mechanisms pathways so that chemicals can be designed with structural attributes that have inherently lower bioactivity potential.

Traditionally, geneticists used forward genetic approaches in zebrafish and other models to characterize and dissect genes involved in biological processes, notably embryonic development (*i.e.*, phenotype-based relationships). These approaches have sought to identify DNA elements involved in biological processes through the screening of populations of organisms exposed to a mutagenizing agent that produces random heritable modifications throughout the genome. Some of the first large-scale forward genetic screens using

zebrafish led to the discovery of a substantial number of shared genes and pathways essential to vertebrate development.[39,40] In contrast, reverse-genetic approaches (genotype-based relationships) involve examining the phenotypic consequences of perturbing the functioning of gene targets. Recent zebrafish genome sequencing/assembly[41,42] and large-scale *in situ* hybridization screens,[43] among other efforts, have revealed thousands of candidate DNA elements that may represent gene targets potentially relevant to chemically mediated bioactivity pathways and thus relevant to chemical design and safety.

In the zebrafish community, morpholino oligonucleotides (MOs) also are a widely used antisense gene knockdown tool to examine toxicity endpoints and disease targets that can be employed early in R&D to design safer chemicals. MOs are ~25 mer nucleic acid bases that are linked to morpholine rings with a neutrally charged phosphorodiamidate backbone that has a high binding affinity to RNA molecules and is stable *in vivo*.[44,45] MO applications in zebrafish can act at the RNA transcript level by inhibiting exon splicing[46] or by blocking translation.[44] Despite their widespread use, MOs have limitations including variability in the degree of knockdown and their transient duration to about three days in early embryogenesis. There is also the possibility for non-specific cell death and other off-target effects, such as p53 inductions, that may produce spurious phenotypes that are not linked to the targeted gene(s) being knocked down. Perhaps most problematic, MO-knockdown approaches in zebrafish have come under increased scrutiny with reports of poor correlation between MO-induced morphants and knockout (KO)-mutant phenotypes.[47–51]

Several genome editing tools have increased the precision of generating targeted mutations in zebrafish that hold promise in clarifying the toxicity pathways and structure–activity relationships of environmental chemicals. The human engineered zinc finger nucleases (ZFNs)[52] and transcription-activator-like effector nuclease (TALENs)[53] were the first methods developed that allowed for the generation of precise heritable mutations. Both methods create site-specific double strand breaks (DSBs) at targeted locations of the genome. These DSBs are then repaired by sequence homology dependent on independent mechanisms that produce targeted mutational edits. While ZFNs and TALENs have been applied in the zebrafish model, their widespread adoption has been constrained by limited multiplexing capabilities and the considerable amount of time and cost required designing the nucleases.[54,55]

The clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system is a newer genome editing tool with potentially broad applications, including in characterizing the genetic pathways involved in toxic responses that would be highly applicable to safer chemical design. The CRISPR-Cas9 system relies on a single guide RNA (sgRNA) and the Cas9 nuclease to generate targeted DSBs next to specific recognition sites called protospacer adjacent motifs that are followed by DNA repair to produce genome edits. The CRISPR-Cas9 system in zebrafish has been used successfully to generate gene KOs,[56,57] disrupt tissue-specific genes,[58] implement single nucleotide substitutions,[59] and introduce exogenous DNA at specific target sites.[60,61] It has been shown to be substantially more efficient at generating germline mutations in zebrafish than the ZFN and TALEN systems.[62] The first HTS CRISPR-Cas9 phenotypic screen of the zebrafish genome targeted 162 loci (83 genes).[62] This screening study reported a 99% success rate in generating somatic mutations with an average germline transmission rate of 28%.

An important attribute that makes the zebrafish such a well suited *in vivo* model for human translational research for chemical discovery and toxicity screening is the capacity to develop transgenic reporter lines that target specific cells/tissue types, molecular signaling pathways, and physiological processes (Fig. 3). Currently, these lines are curated by the Zebrafish Model Organism Database and maintained by ZFIN, University of Oregon (Eugene, OR).[63] This work is contributing to a rapidly expanding diversity of zebrafish disease models and drug/chemical screens to understand, prevent, and treat some of the most recalcitrant and costly diseases of our time, including those linked to: psychiatric conditions;[64,65] cancers;[66–68] diabetes and obesity;[69–71] heart disease;[72–74] neurodegenerative syndromes;[75–78] autism;[79] immunodeficiencies;[80,81] alcohol, tobacco, drug abuse dependency;[82,83] and blood disorders,[84] among many others.

The transcriptome defines the functional and physiological status of an organism and provides information on the gene networks that regulate biological processes. One of the ongoing challenges with zebrafish readouts in chemical toxicity screens is that morphological responses are difficult to decipher because multiple chemicals may elicit the same teratogenic phenotype (*e.g.*, scoliosis, yolk sac edema). Microarray technologies have become useful in hypothesis generating in that they provide an opportunity to dissect toxicological pathways at the transcriptional level in developing zebrafish. Recently, our lab used microarray tools to characterize alterations in the transcriptomes of embryonic zebrafish exposed to the antimicrobial agent triclosan.[85] By phenotypically anchoring the transcriptional alterations to triclosan-mediated developmental malformations, it was possible to propose toxicity mechanisms in zebrafish that may include altered liver development and potential hepatotoxicity. Another related multi-chemical example involved work by Yang and coworkers to apply toxicogenomic methods in embryonic zebrafish to assess whether distinct chemicals could induce specific transcriptional profiles.[86] Gene expression profiles were examined using an oligonucleotide microarray containing 16 399 gene-specific probes. Each of the 11 chemicals tested produced hundreds of genes that were differentially regulated. The expression profiles induced by the chemicals tested were highly specific and could be used similar to a barcode to identify the chemical with high probability. As a final example, to better understand how zebrafish respond to hypoxia, Ton and coworkers used microarray technology to measure the expression changes in >4500 zebrafish genes in embryos

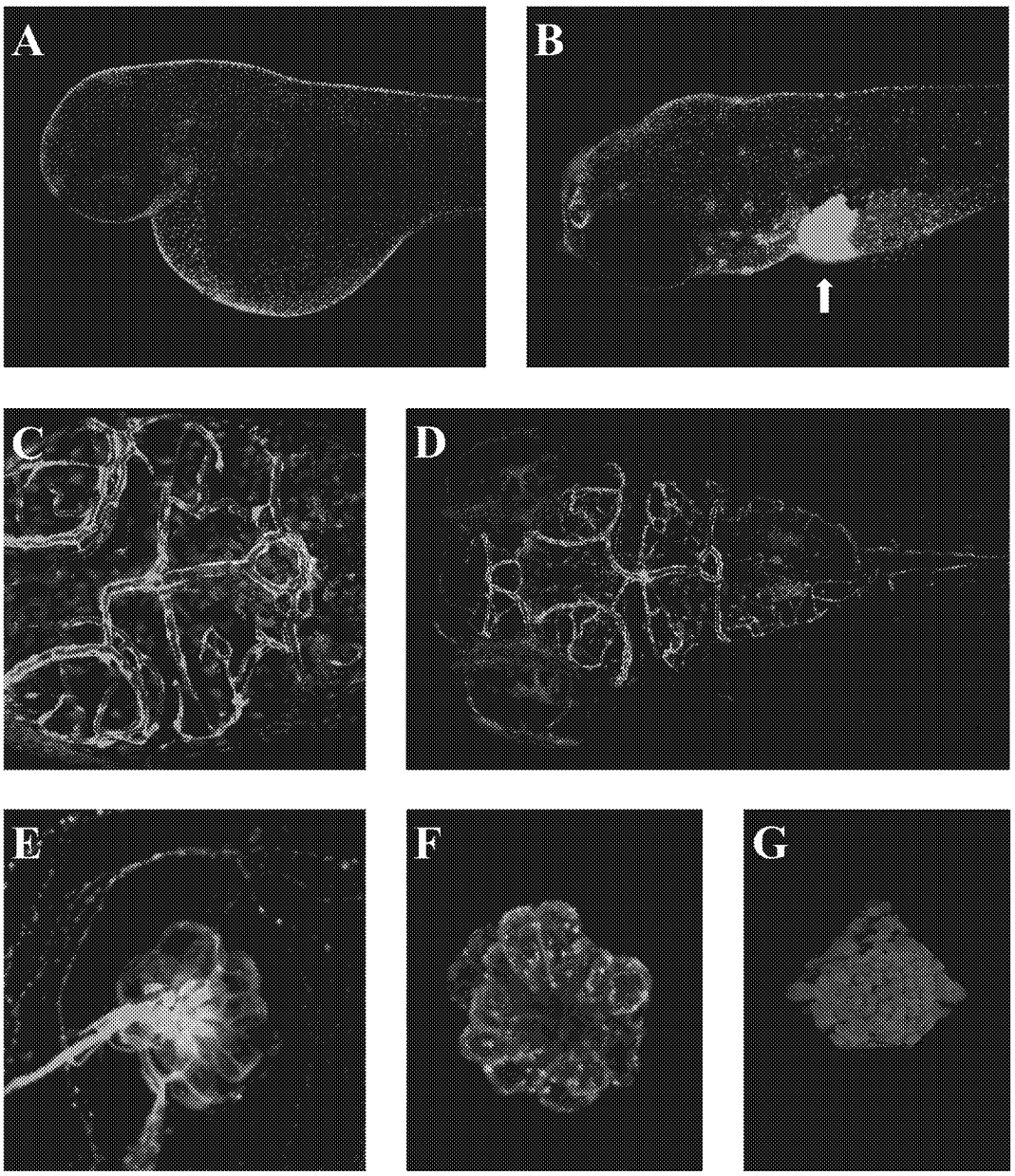

Fig. 3 Examples of embryonic transgenic zebrafish. (A and B) The *Tg(cyp1a:nls-egfp)* line can be used as a surrogate for aryl hydrocarbon receptor (AHR) activity to identify the target organs of chemically exposed larvae. Embryos were continuously exposed to a chemical starting at 6 hpf and imaged at 48 hpf (A) and 120 hpf (B), with noticeable *cyp1a* expression in the liver at 120 hpf (white arrow). (C and D) *Tg(fli:gfp)* embryos, which expresses GFP in endothelial cells of the entire vasculature, were injected with glioblastoma cells (red) into the brain of 4 dpf larvae (C) and reimaged at 7 dpf (D) to understand the invasion and migration behavior of the brain cancer cells in a vertebrate brain microenvironment. (E–G) Immunohistochemistry was used to determine the *in situ* expression pattern of various genes in the hair cells of the lateral line neuromast of 4 dpf larvae. (E) 2D composite image stained with antibodies targeting otoferlin (blue), acetylated tubulin (green), and maguk (red). (F) 2D composite image stained with antibodies targeting otoferlin (green) and vglut3, a synaptic vesicle marker (red). (G) 3D composite image stained with DAPI and the synaptic protein ribeye (red clusters). While images (E–G) are not from a transgenic line, the images were included to highlight the ability to capture high quality *in situ* expression patterns of genes across development, which is the function of transgenic reporter lines.

exposed to 24 hours of hypoxia during development from 48–72 hpf.[87] The downregulation of energy consumption has been shown to be a critical defense mechanism against hypoxia in animals.[88] Results from the Ton study showed a strong coordinated downregulation of genes involved in high energy processes, such as protein synthesis, ion pumping activity, and cell division that were induced by hypoxia and reversed upon re-exposure to normal oxygen conditions. These types of microarray studies provided proof-of-principle that the developing zebrafish can be used as a toxicogenomic model to

systematically asses the effects of chemicals on gene regulatory networks, and with ongoing development can be positioned for use in chemical discovery and the design of safer alternatives.

However, while microarray continues to be popular and transcriptomic methods can be used to characterize the molecular mechanisms of chemical effects, they continue to be hindered by their inability to distinguish direct from indirect effects of a specific treatment. Next-generation sequencing (NGS) technologies, notably RNA-sequencing (RNA-Seq), have emerged more recently in the zebrafish community as an alternative to microarray. RNA-Seq offers several advantages compared with microarrays, such as a more complete snapshot of the transcriptome (*e.g.*, microarrays cannot detect unidentified transcripts and genes), detection of alternative and novel gene isoforms, and coverage of a greater dynamic range at lower abundances. Thus, RNA-Seq appears better suited in discerning unique phenotypes that could be missed with microarray as it is not based on *a priori* knowledge of the transcriptome. Several reports have shown that RNA-Seq outperforms microarrays in the ability to identify significantly differential gene expression by almost 3-fold.[89,90] The increased sensitivity of RNA-Seq is attributed to the improved accuracy for detecting low abundance transcripts. In the zebrafish model, a number of RNA-Seq studies have advanced our understanding of how the transcriptome regulates vertebrate development by identifying paternally- and maternally-derived transcripts,[91] the expressed genes during the maternal to zygotic transition,[92] and the complete set of detectable transcripts across seven different developmental periods.[93] The information provided by these types of studies can be integrated into toxicogenomic reports to provide a vertebrate developmental network in which to identify chemical mechanisms of action. In zebrafish toxicogenomic research, RNA-Seq has been used to reveal conserved biological pathways in 2,3,7,8-TCDD-induced molecular responses in the zebrafish liver compared to *in vivo* mammalian models,[94] identify molecular pathways and biomarkers in response to arsenic exposure,[95] and elucidate the transcriptional responses to oxidative stress in *tert*-butylhydroquinone and 2,3,7,8-TCDD exposed fish.[96]

Improvements in high-throughput genome wide platforms have resulted in a fundamental shift away from protein-centric views of molecular biology. While the number of genes encoding proteins stays relatively constant across a wide range of developmental complexity, the number of non-coding RNA (ncRNA) increases with developmental complexity.[97] Mounting evidence suggests that ncRNAs play significant regulatory roles in complex, multicellular organisms.[98,99] As a result, a number of researchers have looked into the role of ncRNA targets in zebrafish, such as microRNAs, to elucidate their role in toxicity pathways[100,101] or for use as biomarkers of toxicity.[102] In addition to ncRNAs, the expression profiles of proteins,[103] metabolites,[104] and DNA methylation patterns[105,106] are also being investigated in order to develop a more comprehensive understanding of toxicity mechanisms. Continued efforts to integrate omics data into HTS assays will allow for increased efficiency and a deeper understanding of the links between environmental chemical exposures, toxicity mechanisms, and disease, all of which can promote safer chemical design and evaluation efforts.

HTS platforms targeting early development

Chemicals will tend to interact with, and if toxic, perturb multiple targets with effects that may manifest as acute, transient, chronic, or delayed depending on the dose, target, age, and physiological status of the animal in relation to its environment. Moreover, animals may metabolize chemicals to bioactive forms that are more toxic than the parent material, or alternatively may detoxify and excrete chemicals or demonstrate tissue plasticity that may ameliorate adverse effects. Whole organism screens offer the advantage of a more integrated characterization of chemical bioactivity (Fig. 1) thereby avoiding some of the inevitable mechanistic bias of single compound-target pairings and cell-based approaches.

The current state of drug discovery demonstrates some of the advantages conferred by *in vivo* systems in optimizing chemical design and evaluation. Specifically, phenotypic-driven screens with whole animals have demonstrated a higher success rate in identifying promising drug therapeutics than target-based approaches that use *in vitro* and cell culture systems.[107] Although target-based approaches have yielded many thousands of candidate molecules, this has not translated into an increase in drug discovery. About 40% of new candidate molecules fail during preclinical toxicological safety evaluations at great expense and the sacrifice of many test animals.[108] The reasons for the high failure rate of target driven approaches are undoubtedly multifaceted and relate to factors such as the inability to model toxicokinetics and off-target effects in an *in vitro* system. Additionally, these approaches have limited capacity to predict whether modifying a specific target will ameliorate a downstream disease phenotype. These challenges in drug discovery are informative to green chemistry and safer chemical design as they demonstrate some of the limitations of cell-based approaches and the ongoing importance of *in vivo* models, particularly those like zebrafish that fill a niche between *in vitro* and higher vertebrate testing.

An ever-increasing number and variety of low, medium, and higher throughput *in vivo* screens with zebrafish embryos have been and continue to be developed that target an increasing variety of pathways and endpoints (*e.g.*, teratogenicity, endocrine disruption, cardiotoxicity, *etc.*). The implementation of these screening formats with zebrafish has accelerated dramatically in recent years to promote the design of safer chemical alternatives (Fig. 2). While it is not possible to discuss all the many zebrafish assays that have been employed in toxicity evaluations, several approaches are notable due to their relevance to safer chemical design and given the range of chemical structures they aim to consider. They are now being

used to test numerous environmental toxicants, pharmaceutical agents, and chemical libraries across a range of life stages, transgenic and mutant lines, test concentrations, and exposure durations.[109] One of the clear advantages of zebrafish HTS designs is that only very small amounts of test compound in the microgram or microliter range are typically needed, whereas studies in mammalian assays can require upwards of several hundred grams of compound depending on study design and duration. In addition, zebrafish have been shown to be relatively non-responsive to the carrier solvent dimethyl sulfoxide (DMSO).[110] This tolerance to DMSO has made it possible to test an array of chemical structures, including many higher MW chemicals with hydrophobic functional groups that would not ordinarily solubilize in aqueous media. These favorable attributes become especially relevant in early screening of chemical libraries and structures as part of early R&D testing where typically only very small amounts of compound are synthesized with any number of different structural moieties conferring different physicochemical and biological properties.

HTS methods that use embryonic zebrafish are generally consistent with one another in that they use a multi-well plate format to test chemical effects on embryonic development and by assessing mortality and deformities across a range of phenotypes, chemical structures, and concentration ranges (Fig. 4). Medium and higher throughput toxicity screening assays with zebrafish embryos have been developed in the U.S. as part of the U.S. EPA-National Center for Computational Toxicology (NCCT) ToxCast program.[28–30,111] As a part of this effort, for instance, Padilla and coworkers conducted a developmental toxicity study with embryonic zebrafish to screen ~300 chemicals (*i.e.*, mostly pesticides) comprising the Phase 1 ToxCast chemical library.[30] Larvae were scored for survival and overt malformations at 6 dpf. A subsequent study conducted in our lab by Truong *et al.* (2014)[111] used a similar format to the Padilla lab with some differences. Truong *et al.* (2014)[111] evaluated over 1000 chemicals that included the Phase 1 ToxCast chemicals tested by Padilla *et al.* (2012)[30] plus the several hundred chemicals in the Phase 2 ToxCast library. Padilla and coworkers used intact chorionated embryos exposed by static renewal for five days with evaluations on day six, while Truong and coworkers used dechorionated embryos exposed by static non-renewal with evaluations on days one and five. Test concentration ranges were similar, but the Truong study used larger sample sizes and targeted more phenotypes. In terms of screening, Padilla *et al.* (2012[30]) ranked and scored malformations based on severity to calcu-

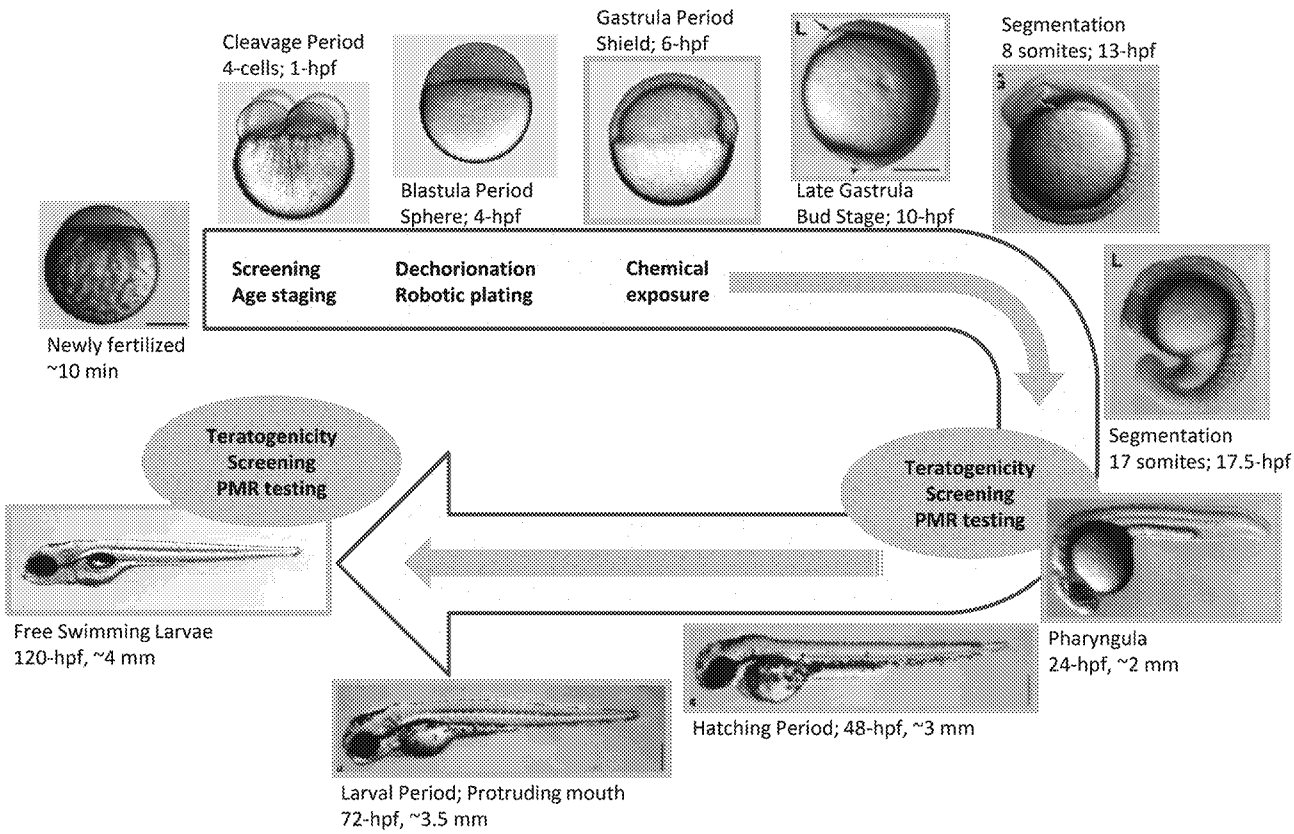

Fig. 4 Example of embryonic zebrafish high throughput screening (HTS) platform. Embryos are life staged, screened for viability, and placed into well plates. Chemical exposures typically occur from 6–120 hours post fertilization (hpf). While chemical screens can occur at different life-stages depending on study goals, morphological evaluations and behavioral assays are conducted often during (1) the early pharyngula stage at 24 hpf when the heart is first clearly visible in a distinct pericardial sac and body/tail flexions initiate with development of the sensory-motor system; and (2) free swimming larvae represented by inflation of the swim bladder, largely completed developmental morphogenesis, and rapid growth.[31,37,40,118]

late half-maximal activity concentration (AC_{50}) values; Truong *et al.* (2014)[111] scored deformities as binary, either present or absent, and computed lowest effect levels (LELs).

While the methods implemented and results in the Padilla and Truong studies digressed both observed similar results. Padilla *et al.* (2012)[30] found that 62% of the ToxCast Phase 1 chemicals were toxic at one or more concentrations at or below the highest concentration tested (80 μM). Likewise, Truong detected toxicity in 60% of the Phase 1 chemical library measured as a positive hit for mortality and malformation across one or more concentrations at or below the highest concentration tested (64 μM). The high percentage of toxic outcomes was expected given that most of the ToxCast Phase I chemicals are pesticides. In comparing positive hits across the two studies, 75% of chemicals scored as toxic in the Truong study were also scored as toxic in the Padilla study, suggesting good concordance across the two platforms but with differences likely attributable to study design. For example, retaining or removing the chorion and variable exposure conditions would be expected to influence the bioavailability and internal dosimetry across the two studies depending on the chemical. Nonetheless, it appears that for chemicals with expected bioactivity, the more limited phenotypic screening by Padilla and coworkers was able to identify chemical-induced developmental abnormalities. Questions remain for compounds with unpredicted or unknown bioactivity that may require more rigorous screening. For example, Truong *et al.* (2014)[111] identified early notochord deformities in embryos exposed to thiocarbamate pesticides that may not have been identified with a more limited phenotypic screen. Thus, there continue to be important considerations as to the breadth and depth of phenotypic screening to balance appropriate rigor (*i.e.*, avoiding false negatives and positives) with maintaining speed and screening capacity.

Similar zebrafish HTS platforms of early development have been used in the design, testing, and evaluation of new chemistries, notably engineered nanomaterials (ENM).[112–117] A major challenge with ENM design centers on identifying features that not only confer desired performance but also minimize toxicity potential. The enormously varied and rapid pace of new ENM structures makes it impractical, absent great time and cost, to conduct extensive *in vivo*-based safety testing without dramatically slowing R&D. Optimizing the biocompatibility of ENM is not a trivial matter as their elemental composition, surface functionality, core size, and purity, among other features, may vary enormously and are technically difficult to characterize. HTS platforms with embryonic zebrafish have shown utility in ENM design as they have been integrated into other platforms intended to characterize the structural features of ENMs. For example, HTS platforms with embryonic zebrafish have been combined with other design methodologies to characterize the physicochemical features (*e.g.*, charge, core size) of different gold nanoparticles (AuNP) that impair development (Harper *et al.*, 2011[114]). Characterizing structural attributes that confer bioactivity can be used as a framework for incorporating safety measures into ENM design to broadly identify structural features in ENM that are not desirable.

These types of screening methods have clear utility in identifying chemicals with heightened or reduced bioactivity that could serve as a useful approach for prioritizing chemicals for more testing and to facilitate the design of safer alternatives. One key challenge with characterizing toxicity results from large, structurally diverse chemical libraries relates to dissecting potentially related responses and common toxicity mechanisms among a data-rich and complex set of phenotypes across a range of structures. To begin to address these challenges, a recent study published by our lab[118] applied the morphometric screening techniques by Truong *et al.* (2014)[111] and integrated results of two locomotor behavioral assays of photomotor responses to characterize the toxicity of over 40 structurally diverse flame retardant (FR) chemicals and their metabolites.[118] Hierarchical clustering and principal component analysis (PCA) were employed to evaluate interactions and differences in bioactivity across the morphological and behavioral platforms to discern chemical classes and structural features that confer elevated bioactivity (Fig. 5). Results of this study measured FR bioactivity in one or more of the assays and across one or more test concentrations, and found that organophosphate FRs with isopropyl, butyl, and cresyl substituents on phenyl rings were especially potent. In sum, this type of integrated HTS approach not only pointed to ongoing concerns for the safety of FRs in use but provided approaches that could be helpful in designing FRs with intrinsically lower bioactivity potential.

Other combinatorial approaches have also been employed to integrate zebrafish HTS with other high content platforms. For example, a large number of organophosphate FRs were recently tested with a battery of HTS platforms that included zebrafish high-throughput methods similar to those conducted in the Padilla, Truong, and Noyes studies, as well as a divergent set of *in vitro* assays.[119] A point of departure (POD) approach was implemented to compare the relative activity of flame retardants tested across the different test platforms. In addition to efforts to rapidly compare effects across multiple platforms, there have been advances in zebrafish technologies that allow for rapid three-dimensional imaging and phenotyping.[120] One of the ongoing limitations of many zebrafish HTS approaches is that most embryonic screening involves the manual examination of chemical effects against a set of developmental defects. This aspect of HTS can be time-consuming and subject to variability depending on the reviewer and lab. There have been promising efforts recently with advanced optical imaging platforms, such as optical projection tomography (OPT), to automate *in vivo* phenotyping of developing zebrafish embryos.[120–122] Ongoing advances in imaging and analysis of different developmental phenotypes should augment the speed and reproducibility of zebrafish HTS platforms.

Ecotoxicology testing

The use of traditional lab models in ecotoxicology has proven to be time and resource intensive, logistically challenging

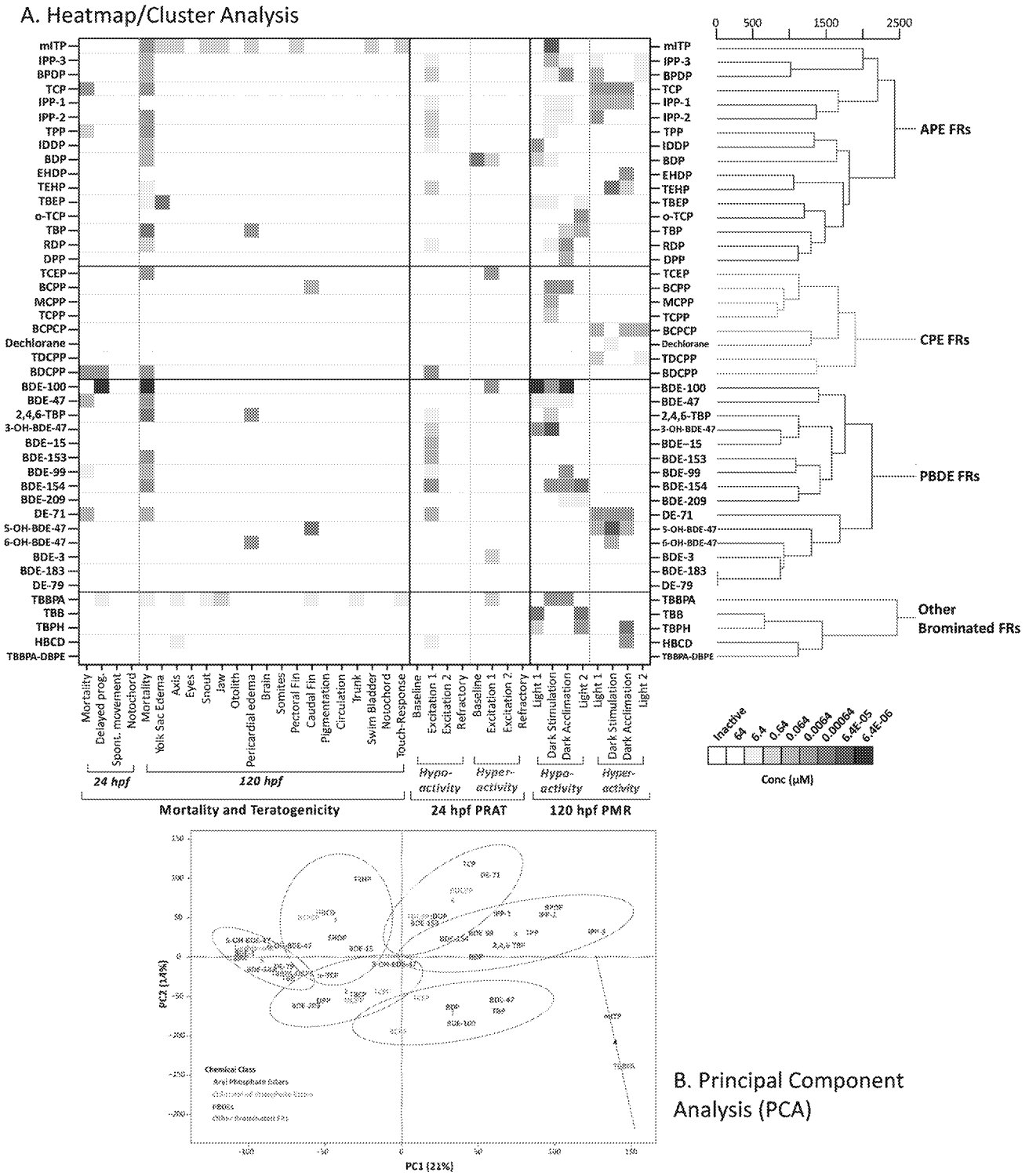

Fig. 5 Chemical structure–activity data analysis of flame retardant (FR) chemicals with embryonic zebrafish high throughput screening (HTS) and photomotor response (PMR) behavioral testing at 24 and 120 hpf, including: (A) heatmap and hierarchical clustering of morphological and behavioral responses (measured as lowest effect levels; LELs) across FR structural groupings; and (B) two-dimensional principal component analysis (PCA) to identify FR clustering patterns based on teratogenicity and behavioral perturbations.[118]

whether conducted in the field or laboratory, and difficult to translate from the lab to wild populations and across species. As a result, like in human health, there has been an accelerating shift from empirical methods to pathway-based methods that rely more on predictive tools and models. Some of these methods seek to characterize putative adverse outcome path-

ways (AOPs) to describe the molecular initiating events (MIEs) and cascades of intermediate key events (KEs) that may culminate in an adverse outcome, such as impaired reproduction or population declines.[25,123,124] In this context, the zebrafish is becoming a useful translational vertebrate model to study chemical bioactivity potential for ecological risk assessment. Perhaps one of the more directly applicable efforts relates to the EU REACH initiative to implement the embryonic zebrafish as a test model to replace fish acute toxicity testing requirements.[26,125] REACH has been the subject of criticism due to the predicted increase in animal testing it triggers and ongoing concerns surrounding how fish experience pain and duress.[126,127] The fish embryo toxicity (FET) screen with zebrafish was developed in part as an alternative model to be responsive to these animal welfare concerns. Additional benefits of FET screens, like with HTS assays more broadly, include that they are efficient and require only small amounts of chemical.

The OECD guidelines for zebrafish FET testing were finalized and adopted in 2013,[128] although FET approaches have been used in Germany since 2005. The guidelines are straightforward and require that newly fertilized zebrafish embryos be exposed to test chemical for 96 hours with microscopic examinations every 24 hours for lethality and other indicators of failed developmental progression, including embryonic coagulation, impaired somite formation, non-detached tail buds from yolk sacs, and lack of a heartbeat. The performance of the zebrafish FET assay in reproducing acute fish toxicity testing results (mined from U.S. EPA ECOTOX and ECETOC Aquatic Toxicity (EAT) databases) was quantified recently for about 140 pesticides, feedstocks, and other chemicals representing a variety of chemical structures.[129] The results measured as half-maximal effect concentration (EC50) values were generally highly correlative across the testing platforms, supporting its adoption for fish ecotoxicity testing. Efforts have been made to try to extend the acute FET assay to include gene microarray analyses for assessing chronic fish toxicity endpoints but overall these tools remain limited to acute endpoints.[130,131] Thus, like with the zebrafish HTS platform more broadly, the FET assay appears to be poised as a useful approach for ecotoxicity applications and chemical design.

The characteristics that make the zebrafish an excellent model for predictive human health assessments are also directly relevant in the context of ecological risk assessment. While historically human health and ecological effects have been assessed using distinct testing methodologies, both disciplines are moving toward predictive approaches that take advantage of our increasing knowledge of biological pathway conservation. Perkins and coworkers recently published approaches that use pathway-based POD data and benchmark dose modeling from embryonic zebrafish exposed to the developmentally toxic pesticide flusilazole to derive human dosing values.[132] These values in zebrafish aligned with those derived from more conventional rodent models and provide some demonstration of how zebrafish can be used to assess chemical risk. The zebrafish may prove to be especially valuable for examining chemical effects on aquatic wildlife because it is increasingly feasible with HTS screens and genetic testing to target multi-chemical exposures and non-chemical interactions.[133–135] For instance, developmental defects leading to cardiac toxicity and heart failure are a well-described sensitive target for the effects of some petroleum-derived PAHs and their mixtures.[135,136] Hicken and coworkers used zebrafish to show how low levels of PAH exposures to embryos interfered with genes involved in heart development that in turn led to reduced swimming performance and changes in cardiac ventricular morphology in adult fish.[133] This mechanistic work with zebrafish is important because it clarified a potential pathway leading from delayed individual toxicity to potentially impaired population fitness among wild fish populations exposed to PAHs by oil spills, hazardous waste sites, and other exposure pathways.

Chemical screens for endocrine activity

Endocrine disruption caused by chemical exposures has been the subject of intensive research and continues to be a concern among many environmental and public health scientists and government agencies.[32,137–143] To date, toxicity studies of potentially endocrine active substances have emphasized the brain-gonadal axis and the brain-thyroid axis. Relatively fewer studies have examined the potential for chemically mediated endocrine activity beyond the gonadal and thyroid axes, and even less have focused on the cross-talk underlying hormone regulation and signaling and how chemicals might interfere with these permissive feedbacks. Some evidence may also support perturbations of the vertebrate endocrine systems at low levels of chemical exposures along with non-monotonic dose–response relationships.[141,144,145] Hormonal systems are involved in many biological responses that are life-stage specific; thus homeostatic perturbations may have profound or transient consequences depending on the age of the organism. The critical importance of thyroid hormone in early fetal development contrasted by the reversible effects (*e.g.*, weight gain) of thyroid hormone insufficiency in adults demonstrates this relationship.[143]

With the rapid advances in genetic testing that allow for characterization of chemical MOAs, the zebrafish has gained prominence in endocrine toxicology as the vertebrate endocrine system, inclusive of the hypothalamus, pituitary, thyroid, pancreas, adrenal gland (fish interrenal organ), ovaries, and testes, are evolutionarily conserved and generally comparable.[146–149] Although important differences exist between reproduction in mammalian and non-mammalian vertebrates, reproduction in jawed vertebrates is controlled by the hypothalamic-pituitary-gonadal (HPG) axis and the structure of this endocrine system is shared. Sex steroid hormones are produced primarily in the gonads of both fish and tetrapods with a synthesis pathway that involves gonadotropin-activated signal transductions, cholesterol mobilization and

transport, and a multistep enzymatic conversion of cholesterol to steroid hormone. Rate limiting steps in steroid production are mediated by production of the steroid acute regulatory (StAR) protein involved in cholesterol transport as well as aromatase, which is a member of the CYP family and regulates estrogen biosynthesis. Both StAR protein and aromatase have been shown to be phylogenetically conserved.[150–152] The general architecture and functioning of the thyroid system is also shared among vertebrates and includes the tightly controlled synthesis of thyroid hormone by the hypothalamic-pituitary-thyroid (HPT) axis and its homeostatic regulation in circulation and target tissues by the activity of iodothyronine deiodinase (Dio) enzymes and other processes. Thus, numerous research efforts have capitalized on this shared biology and used zebrafish to elucidate mechanisms by which chemicals may alter normal endocrine functioning.[153–159]

While it is beyond the scope of this review to describe all aspects of zebrafish use in characterizing chemical-induced endocrine activity, several approaches are highlighted specifically because they demonstrate how this model can facilitate the design and evaluation of inherently safer and more sustainable chemicals. For example, methods involving transcriptomic analyses of embryonic zebrafish have been employed to identify putative estrogen and androgen responsive genes with exposures to hormones and endocrine active synthetic chemicals.[160,161] There are also an increasing variety of zebrafish transgenic fluorescent reporter lines that have been developed to assist in visualizing and characterizing the effects of potentially endocrine active chemicals on brain-gonadal signaling pathways, including *cyp19a*,[162–164] vitellogenin egg precursor protein;[165] growth hormones;[166] estrogen receptors;[167–169] gonadotropin releasing hormone (GnRH) signaling;[170] and glycoproteins encoding follicle stimulating hormone (FSH) and luteinizing hormone (LH).[171] The development of zebrafish transgenic models now extend to other components of the endocrine system that are possible targets of chemicals, including those linked to the brain-thyroid axis,[171–176] endocrine pancreas development and functioning,[177–179] and adrenal-stress responses.[180,181]

Differences in fish and mammal endocrine signaling pathways also can be positioned to further advance our understanding of hormonal and chemical effects on other important biological processes. Questions have been raised about the linkages between neurogenic pathways and aromatase activities in neurological disease, including the role that xenoestrogen exposures may play. Estrogens, in addition to controlling reproduction, have been shown to have extensive and measurable effects on neurogeneration and neuroplasticity in many parts of the brain.[182] Zebrafish have shown a remarkable ability to repair and renew their brains (and other tissues) after traumatic damage in contrast to mammals that have very limited regenerative competencies.[183–185] Moreover, the neural tissues of adult teleost fish have been shown to have 100–1000 fold higher estrogen-synthesizing aromatase activity than in corresponding neural tissues of mammals, including humans.[186] Two distinct genes encoding aromatase enzyme, namely *cyp19a* and *cyp19b*, have been isolated in zebrafish with *cyp19b* being expressed mostly in the brains and *cyp19a* expressed in the gonads.[187–189] Expression of *cyp19b* and aromatase B protein in the brain has been restricted to radial glial cells of adult teleosts.[190] Radial glia are increasingly recognized as progenitor cells that not only are the source of brain neurons during development but are key to ongoing neurogenesis in adult animals.[191] Thus, taken together, the zebrafish may prove to be an important model in exploring the relevance of aromatase and estrogens in tissue repair and regeneration signaling programs, including how endocrine active chemicals and drug agents may hinder or enhance these effects.[191–194]

More broadly, there have been efforts to design protocols that integrate zebrafish testing with other approaches to evaluate the potential for chemicals to interact with the endocrine system. The Tiered Protocol for Endocrine Disruption (TiPED) protocol is one such effort. It describes a step-wise approach, ranging from *in silico* tools to *in vitro* assays and whole organism studies, including with zebrafish, to inform chemical design efforts that minimize endocrine activity.[10] TiPED is intended to foster more sustainable chemical discovery under a non-regulatory framework by providing a tiered methodology for interrogating chemicals for potential endocrine activity. For example, this framework was implemented to evaluate several TAML activators, which are proposed alternatives for water treatment and as oxidizers in breaking down synthetic estrogens and some persistent organic chemicals.[258] As a part of this effort, embryonic zebrafish HTS platforms were implemented to screen these compounds for developmental toxicity. TiPED is an innovative approach for integrating chemistry and toxicology that could serve as a model for guiding chemical design to mitigate against other potentially chemical mediated adverse outcomes (*e.g.*, diabetes, carcinogenicity). It could also be expanded to other endpoints and bioassays that may be indicative of potential chemical bioactivity. For example, zebrafish behavioral assays have also been applied in a limited manner to examine the effects of endocrine active chemicals on possible stress and anxiety endpoints using a 'novel tank' test.[195] The novel tank test has been developed to measure behavioral responses to anxiety (*e.g.*, diving, delayed habituation, thigmotaxis) in zebrafish exposed to chemicals.[195–197] These behavioral assays have been used by Cachat and coworkers to quantify behavioral indices of anxiety in zebrafish (induced by stress or chemical) and to integrate measurements of whole body cortisol.[195] Reider and coworkers employed this type of novel tank test and reported that chemically-induced hypothyroidism with the goitrogen methimazole exacerbated anxiety (latency in exploration/habituation) in zebrafish larvae.[198] In another example, zebrafish behavioral testing has been useful in describing how the potential estrogenic properties of BPA could manifest in a complex array of altered behaviors. BPA is a suspected endocrine active chemical that has been shown in some testing to behave like an estrogen mimic.[199,200] Recent evidence in BPA-exposed zebrafish identified sex-specific differences in behavioral responses

in that male fish were less active (swimming distance, territoriality, and aggression) and circadian rhythms were perturbed in comparison to control males.[201] No significant BPA-linked behavioral effects were reported in females in this study. These types of studies demonstrate the potential for zebrafish behavioral assays to provide a fuller understanding of chemical effects on endocrine-linked anxiety responses and other behavioral domains. However, the results continue to be difficult to interpret. For example, the extent to which anxiety and other behavioral responses in zebrafish are analogous to those of higher vertebrates remains to be clarified. Further studies are also needed to better understand how different zebrafish strains and mutant lines respond in behavioral testing. Another obstacle involves controlling for subtle morphological changes (*e.g.*, musculoskeletal deformities) that might not be detected upon visual inspection but nonetheless could influence fish motor responses. Nonetheless, with continued work, behavioral testing in the zebrafish holds promise in providing a fuller picture of chemical MOAs that proceed in part or substantially through the endocrine system.

Behavioral testing in zebrafish

A number of efforts are underway to develop higher throughput behavioral test methods that use embryonic and larval zebrafish to characterize chemical effects on early neurobehavioral responses that could be highly informative to safer chemical design efforts. One promising area of chemical-behavioral testing involves using embryonic zebrafish (~6 to 120 hpf) as part of developmental neurotoxicity screen to examine chemical effects on early sensory-motor system patterning of the developing nervous system. Starting at 17–19 hpf, zebrafish begin to spontaneously contract their tails reflexively with advancing development of the sensory-motor system.[202] This response has been shown to be highly sensitive and excitatory to light through non-ocular photoreceptors and neuronal pathways activated in the caudal hindbrain and that may involve opsin-based signaling.[203] Targeting this non-ocular response, photomotor response (PMR) platforms with embryonic zebrafish have been designed and validated with large chemical libraries, including approximately 14 000 neuroactive drugs.[202] Briefly, they are rapid assays that involve using a multi-well plate format with chemically-exposed embryos, typically at 24 hpf, and measuring tail contractions and flexions upon short pulses of intense light followed by darkness. Using this type of HTS format and behavioral phenotyping, Kokel and coworkers found that different structural and functional classes of neuroactive chemicals clustered and elicited specific and reproducible behavioral phenotypes in embryonic zebrafish.[202] For example, chemical psychostimulants and anxiolytics increased and decreased motor activity, respectively, throughout the test and regardless of whether light or dark was applied. Dopamine agonists lengthened PMR latency periods, and serotonin reuptake inhibitors showed brief but robust responses to light and even caused stimulated activity to a second light stimulus.

Our laboratory in collaboration has also instituted similar PMR assay designs to test environmental chemicals, including screening the PMR responses of embryonic zebrafish exposed to the roughly 1000 chemicals in the U.S. EPA's Phases 1 and 2 ToxCast libraries.[204] Chemicals that caused light-dependent and -independent effects on embryonic movement in this assay predicted teratogenicity later in older larvae at 5 dpf. In further demonstration of its utility in safer chemical design, this embryonic PMR assay was one of two behavioral assays used recently to test a suite of FR chemicals with variable structural attributes, being integrated into a platform that also measured PMRs and morphometric responses in larvae at 5 dpf.[118] Consistent with observations by Reif and coworkers, the presence or lack of PMR effects in 24-hpf embryos exposed to FRs was predictive of survival and teratogenicity detected later in larvae at 5 dpf. Specifically, the 24-hpf PMR assay predicted the presence or absence of morphological defects for approximately 80% of the FR chemicals examined at 5 dpf. When combined with PMR testing of larvae at 5 dpf, the concordance increased and the presence or absence of 24-hpf and 5-dpf PMR effects predicted 5-dpf teratogenicity for 93% of the flame retardants tested.

Other behavioral screening methods have been applied to take advantage of these earliest movements of embryonic zebrafish. For instance, chlorpyrifos insecticide and other well-described developmental neurotoxicants have been used as training sets to guide and validate embryonic zebrafish spontaneous tail contractions for use in developmental neurotoxicity screening.[205] Raftery *et al.* (2014) used a 384-wellplate format and exposed transgenic embryonic zebrafish (*fli1:egfp*) from 5–25 hpf to 16 chemicals from the U.S. EPA ToxCast Phase 1 library.[206] This study employed enhanced green fluorescent (eGFP) stably expressed in the vascular epithelium of this transgenic line to measure spontaneous tail contractions as an early indicator of developmental neurotoxicity. In this study, tail contractions were absent but no gross morphological defects were observed among embryos exposed to abamectin insecticide from 5–25 hpf. This absence of movement is consistent with other studies showing abamectin neurotoxicity being linked to it agonizing GABA receptors that stimulates release of GABA neurotransmitter and produces paralytic responses.[207–209]

A number of larval zebrafish screens of behavior have been developed, such as measuring preferences, aversions, and locomotion during alternating periods of light and dark. Zebrafish larvae display consistent patterns of visual locomotor activity upon alterations between periods of light and dark, and have been shown to be dark aversive.[210–212] When light is removed a pronounced increase in locomotion occurs that gradually subsides as darkness continues. While the underlying reason for this behavior is still not well described, it has been postulated to be adaptive responses to avoid predators and forage for food. Specifically, evolutionary survival pressures in minnows such as the zebrafish are thought to have given rise to exten-

sive and rapidly developing sensory-motor behaviors, such as saccadic eye movements, optomotor reflexes, rheataxis, startle-escape locomotion, olfactory and feeding behaviors, circadian rhythms, learning, and memory.[213] Thus, the rapidly increasing locomotion observed when larval zebrafish are subjected to darkness has been suggested to be a tractable measure of anxiety, and the decline in movement that is typically observed as darkness continues is proposed to represent habituation.[214–217] PMR assays in zebrafish larvae are now becoming increasingly standardized in application although test regimes may vary depending on the goals of individual studies. Typically, movements of chemically-exposed and control larvae are tracked using a closed box that has a multi-well plate holder, internal lighting system for applying stimuli, and a mounted video camera and software to track and integrate movements for subsequent analysis. These types of larval zebrafish assays have been used in HTS platforms to examine neurobehavioral responses and in some cases underlying neurotoxicity mechanisms for a variety of chemicals, including ethanol,[218–220] nicotine;[221] plastic components and additives;[222,223] nanoparticles,[117,224,225] fluorinated surfactants,[226,227] flame retardants,[118,228–230] pesticides,[231–233] and pharmaceutical agents.[219]

Beyond PMR assays, other locomotor assays and cluster analyses in larval zebrafish have been able to predict targets for chemicals using behavioral profiling. For example, Rihel *et al.* (2010)[216] developed a HTS platform of rest/wake cycles with larval zebrafish and applied it to over 5600 psychoactive drugs to identify important clustering patterns representing relationships between behavioral phenotypes, chemical structures, and biological targets. They showed that neuroactive drugs with different neuro-mechanisms of action (*e.g.*, serotonergic, adrenergic, dopaminergic) elicited distinct behavioral phenotypes. Hierarchical clustering revealed that drugs with correlated behaviors shared common targets and therapeutic mechanisms, allowing in turn for the proposal of targets of chemicals with poorly understood modes of action. For instance, amitraz insecticide, which is used to treat tick and mite infestations in pets and farm animals, clustered with other α2-adrenergic agonist drugs, such as clonidine and guanabenz, reinforcing evidence that this pesticide targets α2-adrenergic receptors and the sympathetic nervous system. Thus, it is clear how continued progress with these types of behavioral platforms could be highly applicable in designing safer chemicals with minimized bioactivity.

Like with developmental life stages of zebrafish, the adult zebrafish has also become a popular model to probe how behavioral and neurobiological endpoints are impacted by chemical exposures. A range of behavioral tests have been designed to target different domains associated with sensory-motor systems, cognitive functioning, and even those more subtle responses related to learning, memory, and anxiety. Indeed, zebrafish adults and juveniles have been shown to display a variety of complex behaviors, such as shoaling and schooling,[234,235] kin recognition,[236,237] territoriality,[238] associative learning,[239–241] and non-associative responses (*e.g.*, habituation);[242] however, as with the neurosciences broadly, our understanding of vertebrate and zebrafish neuroethology and how chemical exposures in turn may cause brain pathologies that produce maladaptive behaviors is an area with many unknowns.

Challenges going forward

While advances in zebrafish testing provide opportunities to predict and characterize chemical structure–activity relationships that promote chemical design for reduced bioactivity, it is important to continually evaluate and choose the most appropriate model for toxicity testing based on research goals and the advantages/limitations of the test organism or assay. For instance, maximizing the use of zebrafish in neurotoxicity testing will require continuing to expand our understanding of the relationships between the structure and function of the CNS and PNS of zebrafish and higher vertebrates. This does not negate the use of zebrafish in characterizing chemical effects on the developing or mature nervous system, but rather points to an area where understanding the homologies and distinguishing aspects of brain and neurological patterning across vertebrate taxa will facilitate a deeper understanding of chemical effects on these pathways and interpreting the results of zebrafish behavioral tests.

Likewise, in addition to some of the characteristics that distinguish zebrafish from higher vertebrates, differences in toxicokinetics and metabolic capacities across vertebrates merit discussion. The rate of absorption, distribution, metabolism, and excretion (ADME) is an important parameter for understanding the bioavailability, internal dosimetry, and ultimately the toxicity of a chemical. *In vitro* studies are limited in that ADME cannot be directly observed. The zebrafish provides a functional system for understanding some of the internal dosimetry and dynamics of chemical exposures. They express the full complement of CYPs seen in higher vertebrates and well-conserved Phase 2 enzyme systems such as transferases involved in endogenous and xenobiotic detoxification pathways.[243,244] Despite these similarities, the metabolic capacity of embryonic zebrafish in comparison to higher vertebrates continues to be an area in need of study. Another related challenge with zebrafish HTS assays is being able to extrapolate a nominal concentration spiked in exposure medium to an internal dose in the embryo and a dose relevant for risk analysis. Absent direct measurement at great cost and time, without knowing the embryonic dosimetry kinetics it is difficult to extrapolate results to a mammalian dose for translation to humans and other higher vertebrates. Moreover, metabolic capacity and toxicokinetics in fish can differ from mammals for some chemical classes that may in turn influence toxicity and targets. For instance, oxidative metabolism of PBDE FRs appears to be only a minor metabolic pathway in fish whereas it dominates PBDE metabolism in mammals producing bioactive hydroxy-PBDE (OH-BDE) metabolites.[245] Another example of these metabolic differences is observed

with some pesticides. Exposures to chlorpyrifos metabolite, chlorpyrifos oxon caused extensive malformations in testing by Truong *et al.* (2014)[111] whereas chlorpyrifos parent was negative for the same endpoints, thereby reinforcing the importance of metabolic considerations in embryonic screens with zebrafish.

Issues pertaining to differential toxicokinetics across species and life-stages raise questions about the concordance of embryonic zebrafish HTS data with toxicity observations in higher level vertebrates (mice, rats, rabbits). There is a growing body of evidence suggesting high concordance between zebrafish HTS and mammalian toxicity results that is consistent with cross-mammalian comparisons and supportive of predictive-based approaches centered on toxicity pathways.[246,247] However, the physicochemical properties influencing toxicity in embryonic zebrafish HTS assays are less clear. For example, in terms of putative chemical uptake, an important discordant result observed between the ToxCast testing by Padilla *et al.* (2012)[30] and Truong *et al.* (2014)[111] pertained to identifying physicochemical properties influencing toxicity. The Padilla study found that toxicity and potency were correlated with chemical hydrophobicity ($\log P$). As the $\log P$ increased for a chemical so too did its toxicity; however, these positive correlations to $\log P$ were not detected by Truong *et al.* (2014)[111] that examined a larger set of chemicals and dechorionated embryos prior to exposure, suggesting that uptake, equilibrium partitioning, and ultimately the toxicity are influenced by the chorion. The zebrafish chorion contains pores that are about 0.17 μm^2 that may contribute to size-dependent exclusion of some larger compounds >3 kDa.[130,248] In at least one study, consistent with Padilla *et al.* (2012)[30], chemical toxicity increased in chorionated zebrafish embryos (48 hpf) with chemical lipophilicity, but overall toxicity was greater in embryos that had been dechorionated.[249] Moreover, in this same study, while the chorion was reported to not play a role in toxicity for hydrophilic chemicals, exposures among dechorionated embryos caused disturbed swimming in larvae that was not observed among exposed chorionated embryos, suggesting that the chorion offered some functionality. Additional work is needed to understand the role, mechanism, and importance of the chorion in influencing toxicity.

Another area where there continues to be research challenges relates to interpreting the readouts of the rapidly expanding diversity of zebrafish behavioral assays. These assays show great promise for understanding chemical effects on animal behavior for translation to humans and by extension safer chemical design. While the power of PMR behavioral profiling as a predictor of chemical structure toxicity holds promise, additional work would help to continue to expand its use by further defining the specificity of the embryonic PMR mechanism (*i.e.*, stimulation of non-ocular photoreceptors) to development of the nervous system (*i.e.*, specificity to developmental neurotoxicity). Moreover, questions remain as to whether the embryonic PMR effects are related to neurobehavioral toxicity pathways *versus* other undetected dysmorphogenesis pathways, or what may be more likely a combination of both neurological and physiological perturbations. Examining chemicals and pharmaceuticals with understood mechanisms and targets with differing potencies would prove beneficial in understanding these relationships. With the large number of transgenic lines, the zebrafish is uniquely suited to characterize how chemical classes and structural attributes target the brain leading to impaired motor and cognitive behaviors.

In addition, the extent to which more recent zebrafish behavioral assays are comparable to mammalian neurobehavioral methods and readouts continues to be a question that will undoubtedly evolve as these test batteries are refined. For instance, unlike rodent and primate behaviors, zebrafish behaviors have not been fully characterized, especially strain-related differences, although progress is being made in defining and cataloging zebrafish behavioral phenotypes.[250] Another ongoing challenge centers on interpretation and specificity, particularly related to translating and anchoring behavioral phenotypes in zebrafish to specific neurological targets.[251,252] These issues also extend to translating behavioral phenotypes measured in zebrafish exposed to chemicals to behavioral responses and targets in higher vertebrates.[250,253,254] There has been work to describe the genes regulating locomotor behavior in larval and embryonic zebrafish.[252] Ongoing challenges related to linking behavioral phenotypes to specific brain pathologies and neurological mechanisms is not singular to zebrafish, but is relevant for all animals models that are trying to understand how chemical exposures may impair or cause maladaptive motor behaviors and impaired cognitive functioning.

Conclusions

It is clear that the zebrafish confers many advantages in toxicity testing that provide an opportunity to optimize safer chemical design and screen the toxicity of the thousands of chemicals already in use. The rapidly expanding variety of genetic assays, HTS technologies, and behavioral test methods that employ the zebrafish model allow scientists to characterize toxicity across multiple levels of biological organization. In combination, it represents a potential data stream rich in molecular, biochemical, functional, and behavioral information that can be positioned for use in sustainable chemistry efforts. The zebrafish as an *in vivo* biosensor provides an opportunity to posit basic questions about the bioactivity of chemical structures early in product design and R&D to discern physicochemical properties, such as functional groups, chemical classes, and chain-length that may confer less or more activity. Though *in vitro* technologies have important application in toxicity screens, the ability to position zebrafish as a bridge between cell-based tools and other *in vivo* models is an exceptional model attribute that allows for extrapolation of data across physiological targets and vertebrate taxa. The translational advantage of the zebrafish is aided by its shared homology to human orthologs. Capitalizing on these attributes, a number of promising HTS tools measuring

bioactivity and behavioral responses are allowing for more automated and rapid 'safety' screens of thousands of chemicals. This initial pass into the chemical space does not provide a great deal of insight into underlying toxicity mechanisms, but can be used to identify more sustainable chemistries. With regard to toxicity mechanisms, though, the zebrafish is an equally important model (*e.g.*, transcriptome profiling, genome editing) catalyzing the shift from empirical tests of chemical effects on apical endpoints (*e.g.*, deformities, survival) to predicting effects on biologically conserved pathways. These tools also provide increasingly meaningful opportunities going forward to characterize the biological effects of chemical mixtures, an area in great need of study. Moreover, these predictive-based approaches are leading to recognition of the integrated connections between human and wildlife health and that the conventional distinctions between human health and ecological risk assessment may not necessarily apply. It is conceivable that the zebrafish could eventually serve as a bridge in future trends to integrate human health and ecological hazard and risk characterizations of chemicals that will be of further use in designing more benign chemicals.

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The authors declare no competing financial interests.

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The views expressed in this manuscript are those of the authors and do not necessarily reflect the views or policies of the U.S. EPA.

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Peer Review Publication 3:

Noyes PD, Lema SC, Macaulay LJ, Douglas NK, Stapleton HM. 2013. Low level exposure to the flame retardant BDE-209 reduces thyroid hormone levels and disrupts thyroid signaling in fathead minnows. *Environmental Science & Technology* 47(17):10012-10021.

Basis for inclusion and scientific impact:

This large laboratory feeding study in fish examined the bioaccumulation, metabolism, and thyroid disruption effects of decabromodiphenyl ether (DecaBDE). I designed this study as part of an EPA STAR graduate fellowship that I was awarded to characterize the toxicokinetics and toxicological modes of action (MOAs) of DecaBDE on the thyroid system of adult and developing fish (Noyes et al. 2011). This is one of the first studies (along with the Noyes et al. 2011 study in fish larvae) to describe the bioavailability and metabolism of DecaBDE in concert with tissue-specific mechanisms leading to thyroid disruption and potential effects on reproduction and development. We were able to predict a debromination pathway in fish based on the suite of lower PBDEs measured in whole fish tissues. Despite the relatively low bioavailability of DecaBDE measured, DecaBDE or its reductive metabolites disrupted thyroid hormone (TH) signaling in adult male fish at multiple levels of the central hypothalamic-pituitary-thyroid (HPT) axis and in peripheral tissues, including causing declines in plasma concentrations of TH, disrupted deiodination of thyroxine (T4) in the brain and liver, and altered the expression of several key genes involved in TH production, transport, and genomic signaling. The brain appeared to be particularly sensitive to DecaBDE-induced hypothyroidism with no apparent tissue-specific compensatory responses observed. The thyroid disruption observed in this study was consistent with effects measured in developing fish of the same species, in which T4 metabolism was reduced, along with substantial thyroid follicle hypertrophy (Noyes et al. 2011). In addition, developing fish exposed to DecaBDE experienced profound liver histopathology presenting as vacuolated hepatocyte nuclei that has been only rarely observed in xenobiotic-exposed animals.

The impact of both these studies has been far-reaching and gratifying. The Noyes et al. 2013 study was a core study used to affirm the risk analysis by the Norwegian EPA to support listing DecaBDE under the Stockholm convention (https://www.informea.org/en/decision/decabromodiphenyl-ether). It was the first study to employ new non-radioactive methods to measure TH concentrations in small volumes of plasma and serum for which I led the development, validation, and publication (Noyes et al. 2013). I worked with Dr. Sean Lema, California Polytechnic University, to isolate and sequence partial cDNA sequences for all three isoforms of the iodothyronine deiodinase enzymes (D1, D2, D3) in fathead minnow. These sequences were the first to be identified in fathead minnow, and I submitted them to NIH's GenBank repository. I presented findings of the adult fish study at the international Flame Retardant meeting in 2013 and was awarded the Åke Bergman/Bo Jansson Award for excellence in presentations. I was also awarded the Hutzinger Award for student presentations at the 2010 Dioxin meeting for my presentation of the Noyes et al. 2011 larval fish study. For both the adult and larval studies, I built the aquatic system, maintained the fish colonies, conducted and managed the exposures, dissections, serum extractions, and analytical measurements, undertook the molecular and toxicity testing, prepared the manuscripts, and managed the journal peer review.

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Low Level Exposure to the Flame Retardant BDE-209 Reduces Thyroid Hormone Levels and Disrupts Thyroid Signaling in Fathead Minnows

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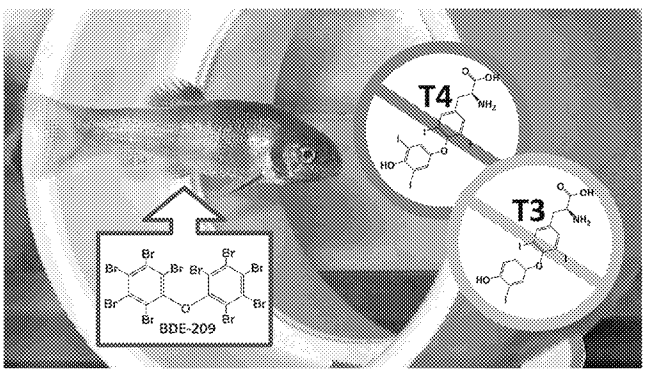
Supporting Information

ABSTRACT: Polybrominated diphenyl ether (PBDE) flame retardants have been shown to disrupt thyroid hormone regulation, neurodevelopment, and reproduction in some animals. However, effects of the most heavily used PBDE, decabromodiphenyl ether (BDE-209), on thyroid functioning remain unclear. This study examined low-dose effects of BDE-209 on thyroid hormone levels and signaling in fathead minnows. Adult males received dietary exposures of BDE-209 at a low dose (~3 ng/g bw-day) and high dose (~300 ng/g bw-day) for 28 days followed by a 14-day depuration to evaluate recovery. Compared to controls, fish exposed to the low dose for 28 days experienced a 53% and 46% decline in circulating total thyroxine (TT4) and 3,5,3′-triiodothyronine (TT3), respectively, while TT4 and TT3 deficits at the high dose were 59% and 62%. Brain deiodinase activity (T4-ORD) was reduced by ~65% at both doses. BDE-209 elevated the relative mRNA expression of genes encoding deiodinases, nuclear thyroid receptors, and membrane transporters in the brain and liver in patterns that varied with time and dose, likely in compensation to hypothyroidism. Declines in the gonadal-somatic index (GSI) and increased mortality were also measured. Effects at the low dose were consistent with the high dose, suggesting nonlinear relationships between BDE-209 exposures and thyroid dysfunction.



INTRODUCTION

The widespread use of the polybrominated diphenyl ether (PBDE) flame retardant DecaBDE has resulted in rising levels of decabromodiphenyl ether (BDE-209) in humans,[1−3] wild fish,[4−6] and other wildlife species.[7−9] BDE-209 is detected increasingly as the dominant PBDE in the atmosphere, sediments, soils, and indoor dust.[3,10,11] These reservoirs of contamination serve as sources of PBDE exposure as BDE-209 can undergo photolytic degradation,[12] microbial breakdown,[13] and metabolic biotransformation[4,14] to lower PBDE congeners. While DecaBDE is scheduled for phase-out in the U.S. at the end of 2013, exposures to BDE-209 and other PBDEs are expected to continue into the coming decades as products that contain them continue to be used, discarded, and recycled. Previous studies have demonstrated that exposures to lower PBDE congeners (e.g., BDE-47, BDE-99) can depress thyroid hormone levels, alter thyroid hormone transport, or target tissue response capacity, and elicit phenotypic impacts such as neurodevelopmental impairments, among other adverse effects.[15−18] Despite the widespread use of BDE-209, however, we continue to have limited information on its potential to impair thyroid functioning. The thyroid system is substantially conserved across vertebrates, and so increasing our knowledge of BDE-209 effects on the fish thyroid can inform our understanding of effects in other species.

This study used the fathead minnow as a model to evaluate effects of low doses of BDE-209 on thyroid functioning and to further elucidate mechanisms of thyroid dysfunction. Two low dose exposures of BDE-209 were selected: (1) a higher dose of ~10 ug/g ww of food targeted to reflect a BDE-209 exposure possible from a more contaminated environment; and (2) a low dose of ~95 ng/g ww food more characteristic of background environmental levels of BDE-209.[19−22] The bioaccumulation of BDE-209 and its reductive metabolites was measured along with effects on circulating thyroid hormone levels, relative iodothyronine deiodinase (Dio) activity and mRNA levels in the brain and liver, and transcript abundances of genes encoding thyroid hormone receptors (*trα*, *trβ*) and several membrane bound transporters from the monocarboxylate transporter (*mct*) and organic anion transport protein (*oatp*) families. Because a limited number of studies have shown

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PBDE impacts on adult fish reproduction,[23,24] the gonado-somatic index (GSI) was measured as an initial metric of BDE-209 effects on reproductive output.

MATERIALS AND METHODS

BDE-209 Dietary Exposures. Approximately 600 adult male fathead minnows (*Pimephales promelas*; 9 months old; Aquatic BioSystems, Fort Collins, CO) were distributed randomly across twelve 150-L glass aquaria (~50 fish/tank) and assigned to the following treatments: three BDE-209 high dose tanks; three BDE-209 low dose tanks; three positive control tanks; and three negative control tanks. Fish at the high dose received dietary exposures (Omnivore Gel Diet; Aquatic Ecosystem, Inc., Apopka, FL) of BDE-209 at 10.1 ± 0.10 μg/g wet weight (ww) of food at 3% bw/day (or ~300 ng/g bw-day). Fish at the low dose were exposed to BDE-209 at 95.3 ± 0.41 ng/g ww of food at 3% bw/day (or ~3 ng/g bw-day). All PBDE concentrations in food were confirmed using mass spectrometry as outlined in the extraction methods below. The model antithyroid drug 6-propyl-2-thiouricil (PTU) was used as a positive control at 0.5 mg/g ww of food at 3% bw/day (or ~15 μg/g bw-day). BDE-209 (97% purity) and PTU were purchased from Sigma-Aldrich (St Louis, MO). Negative control fish received clean food containing cod liver oil vehicle with no BDE-209. Fish were exposed to BDE-209 and control treatments daily for 28 days followed by a 14-day depuration in which fish received clean food containing no test chemical. Fish were euthanized using MS-222 on days 0, 14, 28, and 42 (8–12 fish sampled/tank-sample day). Whole livers, brains, gonads, and plasma were dissected from all fish and preserved at −80 °C for further testing. Fish carcasses were also preserved at −80 °C for PBDE analysis. The sampling and tissue pooling regimen is summarized in the Supporting Information (SI; Table S1) as are the water quality conditions maintained during the study.

PBDE Extractions/Analysis. One fish carcass (dissected of visceral mass, brain, gonad, and plasma) was randomly selected for PBDE analysis across each BDE-209 treatment and control group replicate (n = 3) on each sampling day. Food (BDE-209 amended and control) and fish carcasses were analyzed for a suite of 32 PBDE congeners using gas chromatography mass spectrometry operated in electron capture negative ionization mode (GC/ECNI-MS). The PBDE analytical methods are summarized in the Supporting Information and have been described previously.[25,26] In addition, the Supporting Information (Table S2) reports levels of PBDEs in the BDE-209 amended and control diets.

Plasma T4 and T3 Measurements. Circulating total T4 and T3 (TT4 and TT3, respectively) were measured using a newly developed extraction method[27] and liquid chromatography tandem mass spectrometry (LC/MS/MS).[28] Blood was drawn from the caudal vein of euthanized fish using heparin-coated 75 mm capillary tubes, and centrifuged at 3000 ×G for 5 min to isolate plasma fractions. Plasma was pooled from fish (n = 3; 8–12 fish/replicate). Isotopically labeled hormones, $^{13}C_{12}$-T4 and $^{13}C_6$-T3 (50 μL; 10 ng/mL; Cambridge Isotope Laboratories, Andover, MA; Accustandard, New Haven, CT), were used as internal standards to quantify levels of TT4 and TT3, respectively. Blank controls (deionized water) were extracted alongside samples and were used to correct for trace levels (~0.5%) of unlabeled hormones present as commercial impurities in the labeled standards. Method detection limits (MDLs) and intra/inter-assay %CVs are provided in the Supporting Information.

Deiodinase Activity Assays. Brain and liver microsomes were prepared using previously published methods[25,29] by pooling tissues from six fish per replicate (n = 3; six organs/replicate). Microsomes (1 mg protein) were incubated with 0.64 μM of T4, and formation rates of T3, rT3, and 3,3′-diiodothyronine (T2) catalyzed by Dio enzymes were measured by LC/MS/MS using our previously published methods.[30] All incubations contained 900 μL of 0.1 M potassium phosphate buffer (pH 7.4), 10 mM of dithiothreitol (DTT; Sigma-Aldrich), and 100 μL of the appropriate microsomal fraction diluted to 10 mg/mL. Incubations were undertaken for 1.5 h in a water bath at 25 °C. Negative controls consisted of microsomes incubated with no T4. Labeled internal standards $^{13}C_{12}$-T4, $^{13}C_6$-rT3, $^{13}C_6$-T3, and $^{13}C_6$-3,3′-T2 (100 μL; 250 ng/mL) were added to each sample to quantify levels of T4, rT3, T3, and 3,3′-T2, respectively. Concentrations of thyroid hormones were normalized to time and protein concentration to determine deiodination rates. Blank controls containing buffer alone were used to correct for trace levels (~0.5%) of unlabeled hormones present as commercial impurities in the internal standards. MDLs are provided in the Supporting Information.

Quantitative Real-Time Reverse-Transcribed PCR. Genes encoding the following proteins were targeted for quantitative real-time PCR analysis of brain and liver tissues at each sampling day (n = 6; mean ± SE): Dio enzymes [*dio1* (GenBank accession no. KF042854), *dio2* (KF042855), *dio3* (KF042856)]; thyroid hormone receptors [*trα* (DQ074645); *trβ* (AY533142)]; MCTs [*mct8* (KF053157), *mct10* (KF053158)], and OATPs [*oatp1c1* (KF053149), *oatp1f1* (KF053150), *oatp1f2* (KF053151), *oatp2a1* (KF053152), *oatp2b1* (KF053153), *oatp3a1* (KF053154), *oatp4a1* (KF053155), *oatp5a1* (KF053156)]. Total RNA was extracted from livers and brains of treated and control fish from each sampling day and reverse transcribed to cDNAs using methods summarized in the Supporting Information. Primers and hydrolysis (Taqman) probes were designed to partial cDNA encoding each targeted gene and three reference genes (β-actin form 1, β-actin-1; ribosomal protein l8, *rpl8*; and elongation factor1α, *ef1α*) (SI, Tables S5–S6). A standard curve of serially diluted total RNA (range: 0.049–75.0 ng/μL) from samples representing all treatments and sample days was assayed in triplicate, while half of samples were assayed in duplicate or individually. DNA contamination was assessed for each gene by analyzing samples that were not reverse-transcribed; no amplification was observed. β-actin-1 and *rpl8* were selected as reference genes in the liver and brain, respectively, as neither BDE-209 nor PTU affected their expression. Correlation coefficients (R^2) for standard curves of each gene ranged from 0.98 to 1.00. PCR efficiencies are provided in the Supporting Information (Tables S5–S6) and were calculated using the equation: efficiency = $[10^{(-1/\text{slope})}-1]$.[31] Relative levels of mRNA were calculated for each gene using standard curves and were expressed relative to mRNA levels of the reference gene.[32] Data are presented as values normalized to the negative control at each sample day.

Gonado-Somatic Index (GSI). GSI values for a given replicate tank were derived using published methods[33] and by taking the average GSI of 8–12 fish per replicate on each sampling day.

Statistical Analyses. For the plasma thyroid hormone analysis, the average measurement from three separate extractions was used at each replicate across treatment and sampling day. Differences in circulating thyroid hormone levels were analyzed for statistical significance within sampling day using a one-way ANOVA and Tukey's test (Graphpad Prism 6.0, La Jolla, CA). For Dio activity, differences in thyroid hormone formation rates in T4-incubated microsomes were analyzed within sampling day with a one-way ANOVA and Tukey's test. Changes in gene expression and GSIs in treated and control fish were also evaluated within sampling day using a one-way ANOVA and Tukey's test. For mortality, survival curves were analyzed using a log-rank test; statistical significance was established using a Bonferroni correction for multiple survival curve comparisons. Statistical significance was defined at the $p < 0.05$ level.

RESULTS

Bioaccumulation/Metabolism. Accumulations of BDE-209 and several metabolites, ranging from penta- to octa-BDEs, were measured in both dose groups (SI, Table S7). In the low dose, BDE-209 concentrations increased to 1.4 ± 0.5 ng/g bw at sampling day 14 and then remained relatively stable with concentrations measured at 1.1 ± 0.2 ng/g bw (100 ± 14 ng/g lw) at sampling day 28 and 1.0 ± 0.2 ng/g bw after the 14 day depuration. In high dose fish, BDE-209 concentrations increased from 6.1 ± 1.0 ng/g bw at day 14 to 10 ± 5.4 ng/g bw (2700 ± 1200 ng/g lw) at day 28 after which levels decreased to 3.1 ± 1.0 ng/g bw over the depuration period. BDE-154 (2,2′,4,4′,5,6′-hexaBDE) was the debrominated metabolite detected at the highest concentration after 28 days in both dose groups (1.5 ± 0.1 ng/g bw and 51 ± 7.3 ng/g bw in the low and high dose, respectively), and BDE-101 (2,2′,4,5,5′-pentaBDE) was the lowest molecular weight congener detected (<0.5 ng/g bw and 6.3 ± 0.9 ng/g bw in the low and high dose, respectively).

Plasma Thyroid Hormones. BDE-209 reduced circulating TT4 and TT3 at both doses tested over the 28-day exposure (Figure 1). By day 14, TT3 and TT4 concentrations in the low dose group were significantly reduced by 53 ± 4.1% (1.71 ± 0.26 ng/mL; $p < 0.05$) and 57 ± 6.2% (1.41 ± 0.35 ng/mL; $p < 0.01$), respectively, compared to negative controls (TT3 = 3.67 ± 0.77 ng/mL; TT4 = 3.27 ± 0.41 ng/mL). At day 28, TT3 and TT4 levels in low dose fish continued to be significantly depressed by 46 ± 3.7% (1.62 ± 0.19 ng/mL; $p < 0.05$) and 53 ± 3.6% (1.77 ± 0.23 ng/mL; $p < 0.01$), respectively, compared to negative controls (TT3 = 2.98 ± 0.25 ng/mL; TT4 = 3.73 ± 0.35 ng/mL). Over the 14-day depuration, circulating levels of thyroid hormones in low dose fish remained depressed with TT3 reduced 46 ± 3.7% (1.62 ± 0.19 ng/mL; $p < 0.05$) and TT4 reduced 52 ± 2.8% (1.42 ± 0.14 ng/mL; $p < 0.01$).

At the high dose, significant ($p < 0.01$) declines in plasma TT3 (62 ± 8.2%; 1.13 ± 0.43 ng/mL) and TT4 (59 ± 11%; 1.55 ± 0.73 ng/mL) were measured after the 28-day exposure relative to negative controls (TT3 = 2.98 ± 0.25 ng/mL; TT4 = 3.73 ± 0.35 ng/mL). Over the 14-day depuration, TT3 levels recovered at the high dose, but further reductions in TT4 of 66 ± 3.0% (0.99 ± 0.15 ng/mL) were measured. In the PTU positive control, significant ($p < 0.005$) deficits in TT3 (50 ± 10%; 1.49 ± 0.50 ng/mL) and TT4 (52 ± 10%; 1.81 ± 0.63) were measured at sampling day 28 relative to negative controls. After the 14-day depuration, circulating TT3 levels returned to normal among PTU-treated fish but TT4 continued to be reduced by 59 ± 10% (1.21 ± 0.49 ng/mL).

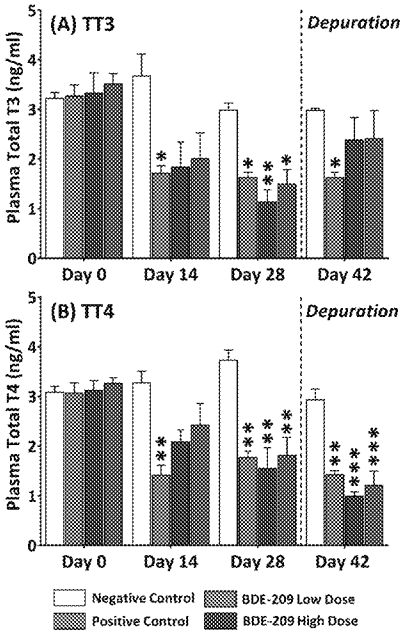

Figure 1. Plasma levels of (A) total T3 and (B) total T4 in adult male fathead minnows exposed to a BDE-209 low dose (95 ± 0.4 ng/g food) and high dose (10 ± 0.1 μg/g food) at 3% bw/day for 28 days followed by a 14-day depuration ($n = 3$; mean ± SE; 8–12 fish/replicate). The model antithyroid agent 6-propyl-2-thiouricil (PTU) was used as a positive control (0.5 mg/g food). Data analyzed within sampling day with a one-way ANOVA and Tukey's test with statistical significance measured at the $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

Deiodinase Activity/mRNA Levels. By sampling day 14, the rate of T4-ORD in the brain (Figure 2A) was reduced by 49 ± 15% (1.37 ± 0.39 pmol T3/h-mg protein), 46 ± 12% (1.44 ± 0.32 pmol T3/h-mg protein), and 44 ± 11% (1.51 ± 0.29 pmol T3/h-mg protein) in BDE-209 low dose, high dose, and positive control fish, respectively, compared to negative controls (2.69 ± 0.19 pmol T3/h-mg protein). By day 28, T4-ORD in the brain had declined further by 65 ± 6.9% and 66 ± 5.0% ($p < 0.005$) at the low dose (1.03 ± 0.26 pmol T3/h-mg protein) and high dose (1.04 ± 0.36 pmol T3/h-mg protein), respectively, compared to negative controls (2.99 ± 0.43 pmol T3/h-mg protein). T4-ORD was also substantially depressed by 74 ± 4.3% (0.76 ± 0.22 pmol T3/h-mg protein; $p < 0.005$) in the PTU positive control at day 28. After the depuration, T4-ORD in brains of BDE-209 and PTU exposed fish returned to negative control levels.

In liver microsomes at sampling day 14 (Figure 2B), T4-ORD increased by 56 ± 7.0% (6.93 ± 0.31 pmol T3/h-mg protein; $p < 0.05$) at the low dose and by 81 ± 16% (8.08 ± 0.73 pmol T3/h-mg protein; $p < 0.05$) at the high dose compared to negative controls (4.46 ± 0.22 pmol T3/h-mg protein). In a reversal, at day 28, the rate of T4-ORD in the liver significantly declined by 29 ± 3.3% (3.90 ± 0.32 pmol T3/h-mg protein; $p < 0.01$) at the low dose and by 42 ± 5.6% (3.20 ± 0.53 pmol T3/h-mg protein; $p < 0.005$) at the high dose relative to negative controls (5.52 ± 0.43 pmol T3/h-mg protein). Similar to the BDE-209 high dose, rates of T4-ORD in PTU positive control fish also declined by 41 ± 5.1% (3.27 ± 0.49 pmol T3/h-mg protein; $p < 0.005$) at day 28. After the depuration, liver T4-ORD in treated animals was not

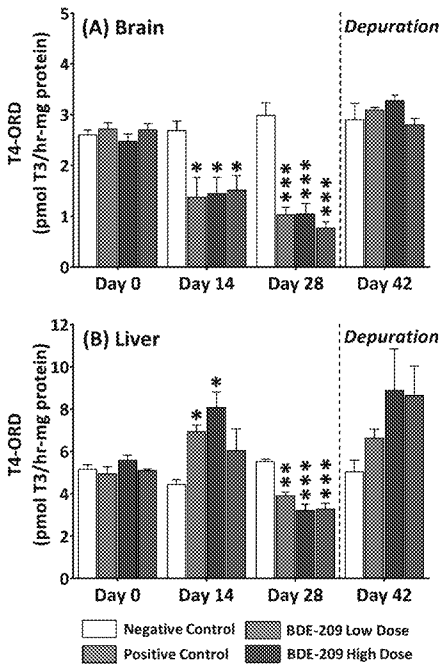

Figure 2. T4-outer ring deiodination in (A) brains and (B) livers of adult male fathead minnows exposed to a BDE-209 low dose (95 ± 0.4 ng/g food) and high dose (10 ± 0.1 μg/g food) at 3% bw/day for 28 days followed by a 14-day depuration (n = 3; mean ± SE; 6 organs/replicate). The model anti-thyroid agent 6-propyl-2-thiouricil (PTU) was used as a positive control (0.5 mg/g food). Data analyzed within sampling day with a one-way ANOVA and Tukey's test with statistical significance measured at the *p < 0.05, **p < 0.01, ***p < 0.005. Note difference in y-axis scales.

significantly elevated from negative controls. No significant changes in T4-inner ring deiodination (IRD) and 3,3′-T2 production (T3-IRD/rT3-ORD) were detected.

At day 14 in BDE-209 high dose fish (Figure 3), relative *dio2* mRNA levels were significantly elevated 12 times in the brain and 4.1 times in the liver compared to mRNA levels in negative controls (p < 0.05). In the low dose at day 14, relative *dio2* mRNA levels were significantly (p < 0.01) elevated 5.3 times in the liver and 4.9 times in the brain compared to negative controls. By day 28, *dio2* transcript levels in treated fish had returned to negative control levels, but relative *dio1* transcripts levels in the brain were significantly (p < 0.05) increased 4.3 times (high dose) and 3.8 times (low dose) that of negative controls. In addition, after the depuration, relative *dio1* and *dio2* transcripts were significantly (p < 0.05) increased 2.9 times that of negative controls in livers of BDE-209 low dose fish. PTU had no effect on *dio* transcription, and no significant changes in *dio3* mRNA levels were detected (SI, Figure S7).

Thyroid Hormone Receptor mRNA Expression. Relative brain *trα* mRNA levels were 2.3 times greater in BDE-209 high dose fish relative to negative controls on day 14 (p < 0.005) and day 28 (p < 0.01) of the exposure (Figure 3C). Moreover, BDE-209 caused a significant (p < 0.05) increase in relative *trα* mRNA abundance in brains of low dose fish at the depuration. Transcription of *trβ* in the brain was not affected by BDE-209 (SI, Figure S7). However, in livers of the BDE-209 low dose and PTU positive control, *trβ* transcripts significantly (p < 0.05) increased to three times that of negative controls (Figure 3F). Transcripts for *trβ* also significantly (p < 0.05) declined at sampling day 28 in livers of BDE-209 high dose fish.

Membrane-Bound Transporter mRNA Expression. At sampling day 14, relative brain *mct8* mRNA levels were about three times higher (p < 0.001) in the BDE-209 high dose and twice as high (p < 0.05) in the BDE-209 low dose and PTU fish than in negative controls (Figure 4A,B). Brain *mct8* transcription returned to negative control levels at sampling day 28, but was again significantly (p < 0.01) elevated about twice that of negative controls after the depuration. Relative liver *mct8* transcript levels were also significantly (p < 0.05) elevated in BDE-209 low dose fish at sampling day 14. No significant changes in *mct10* transcription were observed (SI, Figure S7). Of the *oatp* isoforms tested, only *oatp1c1* transcription (liver

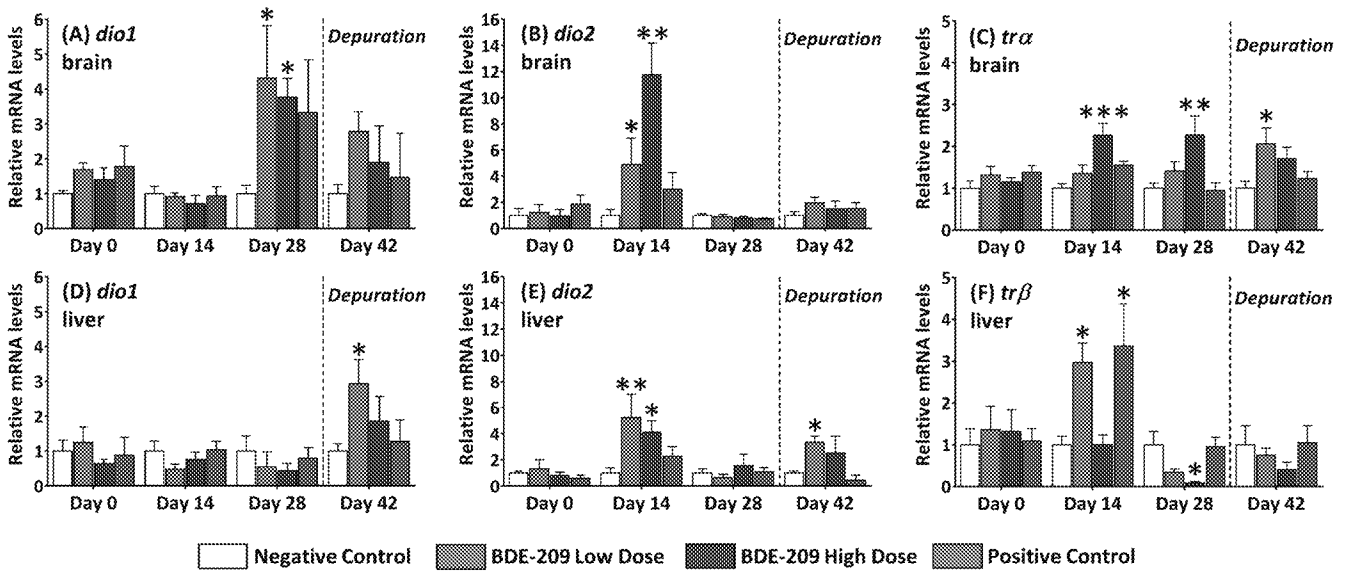

Figure 3. Relative mRNA expression of deiodinases (*dio1, dio2*) and thyroid receptors (*trα, trβ*) in brains and livers of adult male fathead minnows exposed to a BDE-209 low dose (95 ± 0.4 ng/g food) and high dose (10 ± 0.1 μg/g food) at 3% bw/day for 28 days followed by a 14-day depuration (n = 6; mean ± SE). The model antithyroid agent 6-propyl-2-thiouricil (PTU) was used as a positive control (0.5 mg/g food). Statistical significance evaluated within sampling day with one-way ANOVA and Tukey's test (*p < 0.05, **p < 0.01, ***p < 0.005).

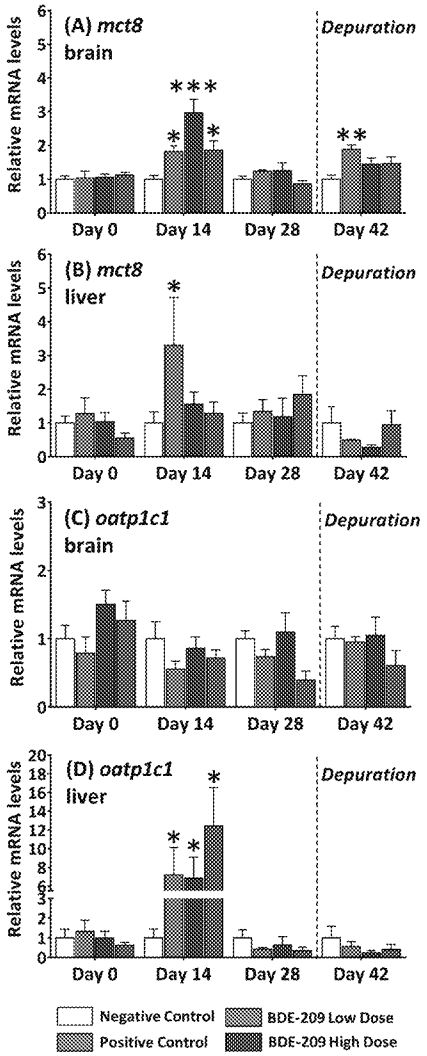

Figure 4. Relative mRNA expression of monocarboxylate transporter (*mct8*) and organic anion transport protein (*oatp1c1*) in brains and livers of adult male fathead minnows exposed to a BDE-209 low dose (95 ± 0.4 ng/g food) and high dose (10 ± 0.1 μg/g food) at 3% bw/day for 28 days followed by a 14-day depuration (n = 6; mean ± SE). The model anti-thyroid agent 6-propyl-2-thiouricil (PTU) was used as a positive control (0.5 mg/g food). Statistical significance evaluated within sampling day with a one-way ANOVA and Tukey's test (*p < 0.05, **p < 0.01, ***p < 0.005).

only; Figure 4C,D) and *oatp2a1* (brain and liver; SI, Figure S8) were significantly affected by BDE-209 exposures. Relative levels of *oatp1c1* increased (p < 0.05) about seven times that of negative controls in livers of both BDE-209 low and high dose fish. In addition, relative *oatp1c1* mRNA levels in PTU treated fish were elevated 12 times that of the negative controls. Abundances for all other *oatp* transcripts tested are provided in the Supporting Information (Figure S8).

Mortality. A statistically significant increase in percent cumulative mortality was measured among both BDE-209 doses. Specifically, 13 ± 3.1% and 12 ± 2.9% of fish from the high and low BDE-209 treatments, respectively, died by the conclusion of the study. We observed <1% mortality in negative controls. Mortality in the PTU positive control group was increased (5.4 ± 2.2%) but was not statistically significant. No significant changes in fish mass, fork length, or condition factor (i.e., fish mass (g)/fork length (cm) × 100) were measured.

Gonado-Somatic Index. Significant declines in the GSI were measured in adult male minnows exposed to BDE-209 at all sampling time points after day 0, including after the depuration (Figure 5). At day 14, the GSI declined 42 ± 13%

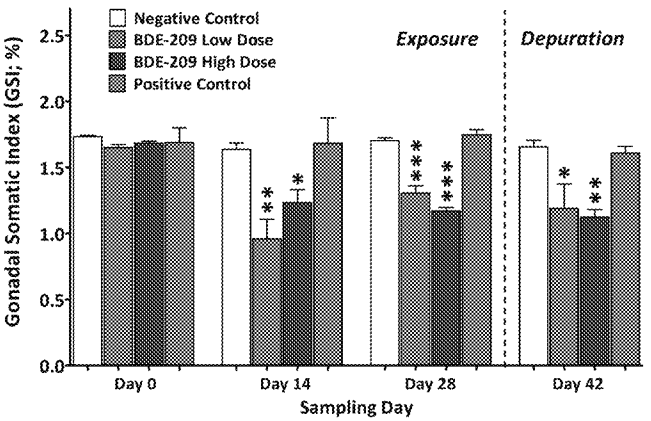

Figure 5. Gonado-somatic index (GSI) measured in adult male fathead exposed to a BDE-209 low dose (95 ± 0.4 ng/g food) and high dose (10 ± 0.1 μg/g food) at 3% bw/day for 28 days followed by a 14-day depuration minnows (n = 3; mean ± SE; 8–12 fish/replicate). The model antithyroid agent 6-propyl-2-thiouricil (PTU) was used as a positive control (0.5 mg/g food). Statistical significance evaluated within sampling day with a one-way ANOVA and Tukey's test (*p < 0.01, **p < 0.01, ***p < 0.005).

(p < 0.01) at the low dose and 25 ± 9.3% (p < 0.05) at the BDE-209 high dose, relative to negative controls. This significantly reduced GSI continued through the end of the 28-day exposure (decline of 23 ± 4.9% at low dose; 31 ± 3.8% at high dose) and extended through the depuration period (decline of 28 ± 15% at low dose; 33 ± 7.0% at high dose). PTU had no effect on the GSI.

DISCUSSION

Bioaccumulation/Metabolism. The BDE-209 bioaccumulation measured here at the low dose (100 ± 14 ng/g lw) and high dose (2700 ± 1200 ng/g lw) is consistent with levels measured in human serum,[1−3,34,35] wild fish, and other wild species[4−6,9,36] (SI, Table S8). Penta- to octaBDE debrominated metabolites measured in adult minnows were identical to metabolites detected in juvenile fathead minnows exposed to BDE-209.[25] Based on the metabolites detected, a pathway of sequential reductive debromination can be proposed (SI, Figure S2) that supports our previous observations of preferential debromination by cleavage of bromine from *meta*-substituted positions.[14,25,37]

Reduced Plasma Thyroid Hormones. The significant deficits in circulating thyroid hormones after the 28-day exposure were consistent with reductions induced by the PTU positive control. PTU is a model anti-thyroid drug that acts primarily at the central hypothalamic-pituitary-thyroid (HPT) axis to reduce T4 by inhibiting thyroid peroxidase iodination of tyrosine residues in thyroglobulin in thyroid follicles. While this is the first study to examine BDE-209 effects on circulating thyroid hormone levels in adult fish, data here are consistent with studies showing that PBDEs, including BDE-209, can elicit reductions in plasma thyroid hormone levels in other species at various life-stages.[23,38−41]

Altered Deiodinase Activity and mRNA Levels. The highly altered brain T4-ORD in adult minnows suggests that thyroid regulation in the brain may be particularly sensitive to BDE-209. T4-ORD reductions in brain microsomes of adult minnows (65 ± 6.9%; Figure 2A) were consistent with declines in T4-ORD (~74%) measured in juvenile fathead minnows receiving equivalent dietary treatments.[25] In contrast to the brain, an increase in T4-ORD was measured in the liver at day 14. This dichotomy suggests tissue-specific differences in regulatory responses to systemic thyroid hormone reductions caused by BDE-209. The elevated T4-ORD in the liver may be attributable to a compensatory response of this tissue to reductions in circulating thyroid hormones. Alternatively, reductions in Dio activity in brain microsomes of treated fish may demonstrate the inability of the brain to compensate locally to depressed levels of hormone. By day 28, T4-ORD was significantly reduced in both the brain and liver, suggesting that any compensatory responses were transient.

BDE-209 effects on T4-ORD were consistent with effects measured in the PTU positive control. In addition to mediating thyroid hormone production at the central HPT, PTU acts in mammalian peripheral tissue by inhibiting Dio1 and is therefore an effective compound to delineate relative Dio activity profiles.[42] However, in fishes, PTU effects on peripheral Dio activity are less clear with some studies suggesting that Dio1 in some species may be resistant to PTU.[43,44] Unlike in BDE-209 treated fish, the PTU positive control treatment had no effect on *dio1* mRNA expression in the brain or liver, suggesting that its effects on minnows were mediated at the central HPT rather than by inhibiting Dio1, while BDE-209 effects appear to be mediated both centrally and in peripheral tissues.

The transient upregulation of *dio1* and *dio2* in response to BDE-209 provides evidence of localized responses of peripheral liver and brain tissues to depressed plasma thyroid hormones. In vertebrates, Dio1 and Dio2 catalyze the T4-ORD pathway to produce the genomically active T3 hormone. This study is one of only a few that has targeted PBDE-induced changes in relative abundances of *dio* mRNA transcription. Consistent with our findings, the relative mRNA expression of *dio1* and *dio2* was increased in zebrafish larvae exposed aqueously to BDE-209[45] and the commercial PentaBDE mixture.[46] An increase in *dio2* transcripts was also measured in livers of larval Chinese rare minnow (*Gobiocypris rarus*) exposed aqueously to BDE-209, although in contrast to our results, a decrease in *dio2* transcripts was reported in brains of adult rare minnows.[47] Finally, the elevated *dio1* and *dio2* mRNA levels in BDE-209 exposed minnows were consistent with studies in which methimazole-induced hypothyroidism increased, while exogenous thyroid hormone decreased, relative *dio1* and *dio2* mRNA levels in livers and brains of fishes.[48−51] Thus, transcriptional regulation of Dios appears to be an important compensatory pathway in teleost responses to BDE-209 and pharmacologically induced hypothyroidism.

Compensatory Responses to BDE-209. The functional and biochemical properties of Dios can provide insights into measured differences in apparent compensatory responses of upregulated *dio* mRNA expression in BDE-209 exposed minnows. In particular, Dio2 has demonstrated substantial physiological plasticity in vertebrates, making it a sensitive regulator of T4-ORD and intracellular T3 homeostasis. It has been shown to be highly sensitive to thyroid hormone with a short half-life in mammals of ~40 min.[52] The early upregulation of *dio2* mRNA expression measured at day 14 in livers and brains of BDE-209 treated fish may be attributable to the rapid homeostatic behavior of Dio2 in response to depressed plasma thyroid hormones. Notably, T4-ORD activity was not increased in brains of BDE-209 treated fish, suggesting that this transcriptional response did not translate to a detectable increase in Dio activity in the adult male minnow brain, although protein levels were not measured.

The absence of a response of brain Dio activity may in part be related to tissue differences in Dio expression. Absolute levels of *dio2* transcript were six times lower in the brain than liver of adult minnows, suggesting that Dio2 activity may likewise be low. Early studies have raised questions about whether *dio2* is expressed in brains of piscivores, as only negligible T4-ORD activity has been measured in the fish brain.[53,54] In accordance with our results, more recent studies using quantitative PCR techniques have localized *dio2* transcripts to the fish brain.[49,55,56] Limited evidence also suggests that the transcriptional response of *dio2* in the brain may be more sensitive to systemic thyroid hormone changes than *dio2* in the liver.[49] Although not known at this time, *dio2* transcriptional responses to BDE-209 may vary with tissue type and could be linked to divergent functional roles of Dio2 in these tissues or to differences in sensitivity to T3.

It is also possible that compensation to prolonged thyroid hormone depression might vary or be more efficient in some tissues than others. The upregulation of *dio1* transcription in the minnow brain at day 28 appears indicative of such regulatory variation under longer periods of hypothyroidism. For instance, no change in relative *dio1* mRNA abundance was observed in brains or livers of parrotfish subjected to experimentally elevated T3 or depressed T4 (by methimazole) for three days.[49] In contrast, *dio1* mRNA transcripts became elevated in two species of tilapia after a 90-day methimazole treatment.[50] Irrespective of the mechanism, compensatory responses of enzymes involved in peripheral thyroid hormone metabolism appear to change over time as BDE-209 exposures continue chronically.

Thyroid Receptors. BDE-209 affected the relative expression of *tr* transcripts in a tissue-specific manner (Figure 3C,F). Two genetically distinct nuclear receptors TRα and TRβ have been identified in fathead minnows with additional subtypes characterized in teleosts.[57−59] Similar to results here, BDE-209 increased *trα* and *trβ* mRNA expression in zebrafish larvae.[45] Aqueous exposures to the commercial PentaBDE mixture, in contrast, had no effect on relative *tr* mRNA abundance in zebrafish larvae.[46] However, thyroid receptor expressional regulation by both thyroid hormones and endocrine disrupting chemicals can vary with fish life-stage.[57−60] The only other study to date that has examined PBDE impacts on *tr* gene expression in adult fish was conducted with BDE-47.[23] In this study, *trα* transcripts were elevated significantly ($p < 0.005$) in brains of female, but not male, fathead minnows exposed to BDE-47, while *trβ* transcripts were depressed ($p < 0.05$) in brains of both sexes. Thus, in addition to age-related influences, there appear to be congener-specific differences in PBDE effects on thyroid receptor expression patterns.

The elevated relative *tr* mRNA levels measured in BDE-209 and PTU dosed fish also reveals an apparent contradiction with studies in hyperthyroid fish. Specifically, an increase in relative *trα* and *trβ* transcript levels has been measured in brains and livers of adult fathead minnows treated with T3.[58] TRs themselves contain thyroid response elements and *tr* tran-

scription can be autoinduced by T3.[60,61] Based on the ability of T3 to induce transcription of its own receptors, a decrease in *tr* mRNA expression might be predicted in BDE-209 and PTU treated fish given the hypothyroidism observed. However, thyroid hormone regulation of TRs appears to be condition dependent. Both hypothyroidism (by thyroidectomy) and hyperthyroidism (T3-induced) have been shown to increase TR expression in the adult rat brain.[62] Other evidence suggests that *tr* transcription can vary within the same tissue[63] and over time as observed in rat brain cell cultures dosed with BDE-99.[64] Thus, our findings of BDE-209 induced elevations in *trα* mRNA abundance in the adult minnow brain at day 14 with altered *trβ* mRNA transcription in the liver might indicate alternative mechanisms of peripheral responses to BDE-209 (and PTU) that have yet to be fully described.

Membrane Bound Transporters. This study is the first to detect impacts of PBDEs on the expression of plasma membrane transporters of thyroid hormones in fish. In both fish and mammals, *mct8* has been structurally and functionally characterized as a specific and active transporter of T4 and T3.[65,66] OATPs mediate the cellular uptake of a range of amphipathic organic molecules, including thyroid hormones and xenobiotics. In humans, OATP1C1 has been found to have relatively narrow substrate specificity with a high affinity for transporting T4 (K_m = 90 nM) and rT3 (K_m = 130 nM).[67] In fishes, however, the diversity of OATPs has only recently been explored in zebrafish[68] and fathead minnows.[69] The observed increases in *mct8* and *oatp1c1* transcript levels in BDE-209 exposed minnows may be characteristic of an upregulation in the expression of these transporters to compensate for the reduced availability of circulating thyroid hormones. This idea is supported by recent findings that T3-treated (hyperthyroid) adult male fathead minnows exhibited reduced *mct8* and *oatp1c1* mRNA levels in the brain and liver.[69] Neither BDE-209 nor PTU increased brain *oatp1c1* mRNA transcripts, despite the substantial hypothyroid status of these fish and the known localization of *oatp1c1* to the fathead minnow brain, ultimately raising further questions about the capacity of the fish brain to maintain homeostasis in cellular thyroid hormone levels under conditions of BDE-209 induced hypothyroidism.

Mechanisms of Thyroid Disruption. Biological effects of BDE-209 on thyroid hormone signaling in the adult minnow proceeded through multiple pathways that involved: declines in circulating thyroid hormones; disrupted T4-ORD in peripheral brain and liver tissues; and altered transcription of genes involved in thyroid hormone production, transport, and genomic signaling. Fish exposed to BDE-209 at both the low and high dose experienced profound deficits in plasma T4 and T3 as well as reduced T4-ORD activity in the liver and brain after a 28-day exposure. While T4-ORD activity recovered after the depuration, circulating T4 (both doses) and T3 (low dose only) remained depressed for at least 14 days after the BDE-209 exposure ceased. Brains of adult minnows appeared particularly sensitive to BDE-209 based on the severely reduced T4-ORD measured in these tissues after 28 days of BDE-209 exposure. Several genes encoding proteins with key functions in thyroid signaling, including *dio1*, *dio2*, *trα*, *trβ*, *mct8*, and *oatp1c1* showed increased expression in BDE-209 exposed fish, although these increases appeared to be transient, compensatory responses to BDE-209 induced hypothyroidism.

Mortality. The increased mortality in this study is not a commonly evaluated end point for PBDEs and has not been observed in previous *in vivo* fish studies conducted in our laboratory.[25,70] However, consistent with our results, a significant increase in mortality (~44%) was measured in adult zebrafish exposed to 1-μM concentrations of BDE-209 for five months, although mortality was also elevated in negative controls (~38%).[71] Fathead minnow adult males exposed to BDE-47 by the diet have likewise shown reduced body condition factors and erratic swimming behaviors, although declines in survival were not reported.[24] No significant changes in body condition were detected in the present study, suggesting that body wasting was not occurring. It is notable, however, that minnows exposed to both doses of BDE-209 displayed greater frequencies of aggressive/territorial behaviors (e.g., fighting, chasing, head-butting) than did negative control and PTU-exposed fish. Thus, while unanticipated, it is possible that BDE-209 mediated shifts in behavior that could have indirectly contributed to increased mortality by increasing physiological stress from social interactions.

Reduced GSI. Few studies to date have evaluated BDE-209 effects on fish reproduction. Consistent with results here, BDE-209 studies in zebrafish have reported altered expression of spermatogenesis genes[47] and decreased GSIs with reduced sperm counts.[71] Adult fathead minnows exposed orally to BDE-47 have also been shown to have decreased mature spermatozoa[23,24] and reduced spawning due to male infertility.[24] Studies in young laboratory rodents have also shown that PBDEs can elicit anti-androgenic effects that impair reproductive development.[72−74] It is notable that in the current study PTU had no effect on the GSI even though thyroid hormones have been shown to influence reproductive functioning.[75−77] This difference suggests that BDE-209 may be impacting adult male reproduction by nonthyroidal mechanisms of action.

Low Dose Effects. The BDE-209 low dose (~3 ng/g bw-day) elicited impacts on thyroid signaling and reductions in the GSI at similar levels to the high dose (~300 ng/g bw-day). Doses tested in this study were generally less than those administered in rodent studies conducted to date with BDE-209.[38−40] Further study is needed to determine whether non-monotonic dose−responses are occurring in fish exposed to BDE-209 as have been detected with other endocrine disruptors.[78] In addition, identification of the congeners driving the thyroid disruption and reduced GSI (i.e., parent BDE-209 and/or its metabolites) was beyond the scope of this study but merits further investigation. The thyroid system is well-conserved across vertebrate taxa, and our findings that BDE-209 exposure at low doses can impact thyroid hormone homeostasis and signaling at several levels point to a need to further evaluate the potential for BDE-209 induced thyroid dysfunction in humans and wildlife.

■ ASSOCIATED CONTENT

Supporting Information

Descriptions of PBDE analytical methods, isolation/sequencing of partial cDNAs, quantitative PCR methods, primer/hydrolysis probe sequences, PBDE bioaccumulation/metabolism, MDLs and intra/inter-assay %CVs of plasma thyroid hormone/Dio activity, and relative mRNA transcript levels of *dio3*, *mct10*, and *oatps*. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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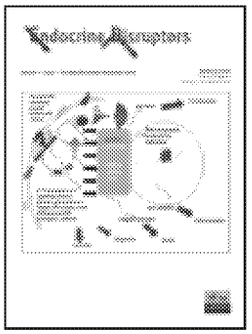
Peer Review Publication 4:

Noyes PD, Stapleton HM. 2014. PBDE flame retardants: Toxicokinetics and thyroid endocrine disruption in fish. *Endocrine Disruptors* 2: 2943001-2943025.

Basis for inclusion and scientific impact:

This paper updated the state-of-the-science on the biological effects and toxicity mechanisms of the PBDE flame retardant chemicals, which despite their use restrictions and phase-out in many countries, continue to be pervasive in humans and the global environment. I was the co-lead on this paper with my PhD advisor, Dr. Heather Stapleton, Duke University, who is a leading environmental chemist in the field and has published some of the foundational studies describing levels, metabolism, and effects of flame retardants in people and wildlife. I wrote the paper and conducted all associated analyses, including the extensive literature survey that was undertaken; Dr. Stapleton reviewed the paper and provided a number of useful comments and text edits. The review updated the evidence for PBDE toxicokinetics and toxicity mechanisms in teleosts, leading to perturbations to the thyroid axis and impairments in development and reproductive functioning. It is a unique assessment in that it was written with an eye toward evidence of mammalian toxicokinetics of the PBDEs, and their associated effects on human health. It included extensive analysis of the similarities and differences in PBDE metabolism and toxicological effects observed in mammals and teleosts given, for example, the high level of evolutionary conservation of the vertebrate thyroid system and how this translates to common molecular targets and biological responses. I conducted an in-depth analysis of the leading toxicological modes of action of PBDEs on the vertebrate thyroid system, and evidence of associated adverse outcomes on early development. Another important contribution of this review was that it is one of the first reviews to examine and synthesize the evidence for PBDE effects on reproduction. It also remains one of the only reviews to examine the interactive links and role of thyroid axis perturbations on the estrogen and androgen pathways. Though the journal Endocrine Disruptors was only established in 2013, to date, this is the fourth most cited article in the journal and the fifth most read and downloaded at over 890 times.



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PBDE flame retardants

Toxicokinetics and thyroid hormone endocrine disruption in fish

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Keywords: endocrine disruption, feminization, flame retardant, neurodevelopment, neurotoxicity, polybrominated diphenyl ether, reproduction, thyroid hormone, thyroxine, thyroid receptor

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardant chemicals that have been used in large quantities and are now detected worldwide in humans and wildlife. To complement reviews of effects on human health, this review discusses and synthesizes current evidence of PBDE toxicokinetics and toxicity mechanisms leading to perturbations of thyroid hormone homeostasis in fish. PBDE disruptions to thyroid signaling in fish appear to proceed through multiple pathways involving declines in circulating thyroid hormones, disrupted deiodination activity, hindered hormone transport, and altered transcriptional regulation of genes involved in thyroid hormone production, transport, and genomic signaling. PBDE exposures have also been linked to impacts on reproductive health with reductions in fecundity, spawning, hatching success, and offspring survival observed in some species, as well as impaired fertility. These studies on PBDE mediated hormone disruption in fish can help inform future studies seeking to understand potential developmental effects in humans.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardant (BFR) chemicals that have been added to many consumer and commercial products including textiles, carpeting, construction materials, and electronics in an effort to reduce their combustibility. Although these compounds have been both banned or phased-out from production in a number of countries, human and environmental exposures continue as products that contain these chemicals are still used and recycled, and present legacy contamination problems upon disposal. As a consequence, PBDEs are widespread and persistent contaminants in both living and non-living parts of the global environment.[1-9]

PBDEs can have from 1–10 bromine atoms substituted on diphenyl ether (Fig. 1). There are 209 PBDE congeners (BDE-1 to BDE-209) theoretically possible depending on the number and substitution patterns of bromine. In practice, however, the number of congeners formed is limited based on the chemical properties and composition of the PBDE commercial mixtures. Three PBDE commercial mixtures have been produced: PentaBDE, OctaBDE, and DecaBDE. The PentaBDE commercial mixture is a heterogeneous mix of tetra-, penta-, and hexaBDEs and was added mostly to polyurethane foams and textiles, and to a lesser extent in epoxy and phenolic resins and polyesters. The majority (~95%) of PentaBDE was used in the US where it was added to polyurethane foams in furniture cushioning and could constitute up to 30% by weight of these products.[10] The OctaBDE commercial mixture is made up of higher molecular weight (MW) constituents, hepta, octa, and nona-BDEs and was added predominantly to acrylonitrile butadiene styrene (ABS) used in the plastic housing of office equipment and electronics. The production and use of PentaBDE and OctaBDE were phased out in the US and banned in the European Union in 2004 due to concerns about their persistence, bioaccumulation, and toxicity. In 2009, these products were also listed as persistent organic pollutants (POPs) under the United Nations Stockholm Convention.[11] The DecaBDE mixture contains the fully brominated congener decabromodiphenyl ether (BDE-209; ~97%) with trace amounts of nonaBDEs. It is used as an additive in high impact polystyrene, polyolefin, and polypropylene plastics used in electronics, automobiles, airplanes, and construction and building materials. To achieve US fire safety standards for plastics, high impact polystyrene plastics typically contain ~10–15% of DecaBDE.[12] DecaBDE is one of two dominant BFRs used worldwide with 2007 global consumption estimated at ~73 000 t (~161 million pounds).[13,14] The production of DecaBDE was discontinued in the US at the end of 2013 under a voluntary phase-out and has been restricted from use in electronics in the European Union since 2008. It is generally unrestricted from use in Asia.[15]

PBDEs structurally resemble polybrominated biphenyls (PBBs), polychlorinated biphenyls (PCBs) and some biomolecules, most notably thyroid hormones (THs; Fig. 1). Because PBDEs are not chemically bound but are rather added to plastics, they can enter the environment during production and may be released into the surrounding environment and biota with the breakdown and volatilization of the parent polymer. Like other persistent, hydrophobic chemicals, the most important route

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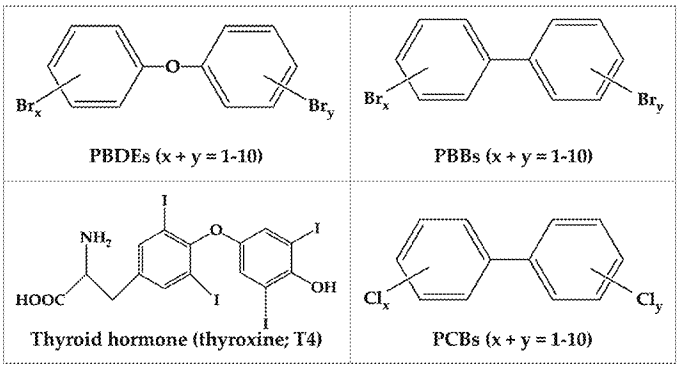

Figure 1. Structural comparison of PBDEs, PBBs, PCBs, and thyroxine hormone

of uptake in aquatic animals appears to be by trophic transfer and consumption of foods contaminated with PBDEs.[16,17] This dietary exposure pathway in aquatic animals is distinguished from human uptake that appears to depend on both dietary exposures and the incidental ingestion of PBDE-containing dust.[18,19]

Despite the discontinued use of PentaBDE, its constituents, including BDE-47 (2,2'4,4'-tetraBDE), BDE-99 (2,2',4,4',5-pentaBDE), BDE-100 (2,2',4,4',6-pentaBDE), BDE-153 (2,2',4,4',5,5'-hexaBDE), and BDE-154 (2,2',4,4',5,6'-hexaBDE), continue to be dominant PBDEs frequently detected in humans and wildlife worldwide despite the generally more limited use of PentaBDE outside the US.[20,21] Potential sources of these congeners are likely related to the ongoing use and recycling of products that contain PentaBDE as well as their high environmental persistence and long-range global transport potential.[22] Another source of these lower MW PBDEs may be attributable to the breakdown of higher PBDEs, such as BDE-209, which can undergo photolytic degradation,[23] microbial breakdown,[24] and metabolic biotransformation[25] to lower MW congeners.

Recent attention has focused on the potential for BDE-209 and other highly brominated PBDEs to bioaccumulate. BDE-209 is now the dominant PBDE measured in abiotic compartments, typically at ppb to ppm levels (ng/g dry weight [dw] to low mg/g dw) in dust,[18,26] soils and sediments,[27-29] and biosolids.[30,31] Studies show that BDE-209 is bioaccumulating in a large number and variety of biota residing all over the world, including, for example, in birds and bird eggs,[32,33] terrestrial and aquatic mammals,[34-36] and plankton, fish and shellfish.[20,37] Human body burdens of BDE-209 also appear to be rising including among E-waste workers and people residing near PBDE production facilities[38,39] as well as among the general population, particularly young children in the US.[18,40] People residing in some regions of China with heavy E-waste recycling operations report some of the highest PBDE body burdens in the world, with BDE-209 concentrations in serum at greater than 3000 ng/g lipid. DecaBDE is now more commonly detected among the general population, particularly young children in the US population. For example, recent work in our laboratory,[18] in collaboration with the Centers for Disease Control (CDC) and Boston University, measured BDE-209 levels in the serum of a North Carolina cohort of toddlers (3 y old) ranging from <6–68 ng/g lipid. In addition, other highly brominated PBDEs are being detected more frequently in biota, including the hexa- to nonaBDEs, which may reflect the increased use of BDE-209 and its environmental breakdown and biological metabolism.[17,41]

This review summarizes the biological disposition and toxicity of PBDEs in teleost fishes with particular focus on thyroid disruption mechanisms and interactions as this endocrine system is an important target of the PBDEs. Indeed, most laboratory studies published to date in fish (and other vertebrates) have focused on the potential for PBDEs to perturb thyroid signaling, as well as impair neurological development and reproduction. While these are often the primary endpoints of focus, other toxicity outcomes have been observed in fish as well, including immunotoxicity[42,43] and oxidative stress.[44-46] The PentaBDE commercial mixture and its component congeners have been the subject of most study to date, with less known about the toxicity of BDE-209 and the other higher MW PBDE congeners. However, BDE-209 is the only PBDE that has been evaluated for carcinogenicity with "suggestive evidence of carcinogenic potential" based on increased thyroid cell hyperplasia and thyroid adenomas/carcinomas in male mice and liver tumors in male rats.[47]

PBDE data generated in fish not only informs our understanding of potential effects in wild fish species and populations but also provides important information on mechanisms of toxicity and disease in humans due to the high degree of gene and functional conservation shared across vertebrates. Indeed, PBDE toxicity measured in fish shares mutual features and effects to those observed among in vivo and in vitro mammalian models, supporting potentially common biological mechanisms of toxicity that also lend biological plausibility to the human epidemiology data on PBDEs. Therefore, this review includes brief discussion of effects observed in rodent and human epidemiology studies in so far as to frame a fuller picture of the toxicity of these chemicals. PBDE human health effects have been examined in several informative reviews and readers are referred to these papers for more detailed analyses.[48-50]

Toxicokinetics of PBDEs

Patterns of PBDE toxicokinetics (i.e., absorption, distribution, metabolism, and excretion; ADME) in fishes have been shown to vary depending on the PBDE congener, species, life-stage, and route of exposure. Table 1 summarizes PBDE toxicokinetic studies in fishes to date.

PBDE Uptake and Tissue Distribution

Our understanding of PBDE absorption in fishes is somewhat limited by the species and PBDE congeners studied. Two early studies in Northern Pike (*E. lucius*) found that BDE-47 was readily absorbed with measured uptake efficiencies of ^{14}C-BDE-47 and unlabeled BDE-47 at 90–100%.[51,52] This research group also measured uptake efficiencies of BDE-99 and BDE-153 in pike at

~60% and ~40%, respectively. Studies in rodents have likewise measured absorption of BDE-47, -99, -100, -153, and -154 in the range of ~70–90%.[53-55] However, other studies that have exposed fish to unlabeled PBDEs through the diet have measured lower assimilation efficiencies of some congeners, including BDE-99, BDE-153, BDE-183, and BDE-209 suggesting species-specific differences in assimilation efficiencies of PBDEs possibly due to differences in metabolic enzyme systems.[56-58] For instance, BDE-209 absorption in some teleost fishes has been shown to occur at a slow rate, which may allow for greater metabolism and elimination than seen in terrestrial species. A dietary study in juvenile rainbow trout receiving 7.5–10 mg/kg bw-day of BDE-209 measured bioavailability at <1%[59] with higher bioavailability of 3.2% measured in juveniles of the same species receiving a chronic dietary exposure.[60] In fathead minnow juveniles and adults exposed orally to ~10 μg/g ww food, BDE-209 uptake efficiencies were also low at 5.8%[61] and 1.3%,[62] respectively. Nonetheless, despite its low bioavailability, BDE-209 appears to be bioaccumulating in fish as shown in field measures and laboratory-based studies with BDE-209 spiked sediments.[6] While the apparent dichotomy between low bioavailability of some PBDEs, notably BDE-209, and observed bioaccumulation are not fully described, rainbow trout exposed to BDE-209 thru the diet have been shown to bioaccumulate BDE-209 at 1.3 times the concentration of levels in their food, suggesting bioaccumulation that is strongly influenced by tissue composition.[60] In addition, recent findings in Chinese sturgeon (*A. sinensis*) also support that tissue distribution patterns of the higher PBDEs, rather than lipid binding, are important factors influencing their bioaccumulation.[63] This study showed low partitioning of the higher PBDEs (heptaBDEs to BDE-209) from blood to tissues that could in turn lead to their slower delivery to metabolically active tissues and thus higher bioaccumulation.

The dominant PBDEs measured in biota (i.e., BDE-47, -99, -100, -153, and -154) are deposited to lipophilic tissue compartments, and these congeners continue to be detected in human serum, breast tissue, and milk[18,64,65] and in adipose tissues of a variety of wildlife, including free ranging fish species.[17,66,67] PBDEs have been shown to cross the blood–placenta and blood-brain barriers to accumulate in the brains of perinatally exposed rats exposed to the PentaBDE commercial mixture[68] and some birds of prey.[69] In addition to accumulation in lipid-rich tissues, the liver is an important target of PBDE disposition and toxicity. The US EPA National Toxicology Program has published findings showing hepatotoxicity, including elevated liver enzyme activity accompanied by hepatic hypertrophy and vacuolizations in mice exposed orally to the PentaBDE mixture for 13 wk.[70] The liver also appears to be an important site of PBDE accumulation in fish. In pike fish, ^{14}C-BDE-47 accumulated in the liver and in lipid rich tissues. In rainbow trout (*O. mykiss*) exposed orally to BDE-209, the highest concentration of BDE-209 was measured in the liver on both a lipid normalized and body weight basis, followed by accumulation in the serum, with less accumulation in the carcass.[60] BDE-209 deposition to adipose tissues might be predicted given its low water solubility (<0.1 μg/l) and high octanol-water partitioning coefficient (log $K_{ow;}$ 6–12).[71] However, studies have shown that this pattern of preferential deposition to lipid depots does not occur substantially for BDE-209. Rather, BDE-209 preferentially distributes to highly perfused, blood-rich tissues, particularly the liver, kidney, heart, and intestinal wall.[25,72,73] The underlying reasons for this distribution pattern appear to be attributable to the large size of BDE-209 and its ability to bind with plasma proteins.[72]

PBDE Reductive Metabolism in Fish

To understand PBDE metabolic pathways in fish, it is informative to frame the discussion in terms of present knowledge of PBDE metabolism in mammals as this is now fairly well described. The rodent literature supports a PBDE metabolic pathway in mammals that has two major reactions: (1) a cytochrome P450 (CYP450)-mediated epoxidation of PBDE phenyl rings catalyzed predominantly by CYP2B (by constitutive androstane receptor [CAR] inductions) and by CYP3A (by pregnane X receptor [PXR] inductions); and (2) debromination or Phase II conjugation of an OH-intermediate with glucuronides catalyzed by uridine diphosphate glucuronosyl transferases (UDPGTs) and with sulfates by sulfotransferases (SULTs).[87,88] Reductive debromination reactions appear to be minor pathways of PBDE metabolism in mammals (e.g., BDE-209 debromination to octa- and nonaBDEs).

PBDE metabolism in teleost fish appears to be different from that in mammals. As outlined in **Table 1**, a large number of studies have shown reductive debromination of PBDEs to be a major route of metabolism, including in common carp (*C. carpio*),[25] fathead minnow (*P. promelas*)[61,62]; rainbow trout,[59,60] lake trout (*S. namaycush*),[89] Chinook salmon (*O. tshawytscha*),[76,83] and zebrafish (*D. rerio*).[78] However, while PBDE reductive debromination appears to be a major metabolic pathway in fish, the role of specific enzyme systems in catalyzing this biotransformation remains unclear. One pathway that has been hypothesized to mediate PBDE reductive metabolism in fish is by the activity of iodothyronine deiodinase (Dio) enzymes.[25,89] Dios are membrane-bound enzymes that are expressed on plasma membranes and in the endoplasmic reticulum, and regulate TH levels in vertebrates.[90] There are three known Dio isoforms in fish, Types 1, 2, and 3 (Dio 1, Dio 2, and Dio 3, respectively) that share functional homology with mammalian Dio isoforms. As illustrated in **Figure 2**, the conversion of the TH thyroxine (T4) to the genomically active 3,3′,5-triiodothyronine (T3) hormone is catalyzed by the cleavage of iodine from the *meta*-position of the outer phenyl ring of T4. The reductive debromination of PBDEs in fishes is also dominated by *meta*-cleavages of bromine, suggesting a possible role for these enzymes in catalyzing PBDE debromination.[25,89] More recent studies have shown that the reductive debromination of BDE-99 to BDE-47 can be substantially inhibited by co-incubating liver microsomes from common carp with THs, suggesting that BDE-99 may be a substrate that competes with THs for Dio enzyme activity.[74,81]

Although BDE-glutathione metabolites have been measured in rodents[53,91] and birds,[92] glutathione-*S*-transferases (GSTs) have

Table 1. PBDE toxicokinetics measured in teleost fish species

Species	Treatment	Route	Dose/Duration	Effects Observed	Ref
Common carp (Juv.; *C. carpio*)	BDE-99	GI, liver microsomes	12–29 pmol/mg protein; 60 min incub	Reductive debromination: BDE-99 to BDE-47 Metabolism in liver > intestine No debromination by GI microflora	74
Atlantic salmon (Juv; *S. salar*)	PentaBDE; OctaBDE	Diet	10, 50 mg/kg bw; 7 d	No significant hepatic CYP1A induction or protein expression	75
Chinook salmon (Adu.; *O. tshawytscha*)	BDE-99	Liver microsomes, cytosol	0.03 - 1.8 µM; 16 h incub	Reductive debromination: BDE-99 to BDE-49 negative GST/CDNB assay	76
Northern Pike (*E. Lucius*)	PCBs, PCNs, BDE-47, -99, -153	Diet	90 ng/µl lipid (10 µl injected into rainbow trout); 9 d	Uptake efficiencies: BDE-47 = ~90%; BDE-99 = ~60%; BDE-153 = ~40%	51
Northern Pike	^{14}C-BDE-47	Diet	16.2 µg/µl; 9, 18, 36, 65 d	^{14}C-BDE-47 uptake > 90% Disposition: Highest in liver, adipose tissue, spinal cord-surrounding tissue, eyes, gall bladder; Lowest in muscle, spleen, gills	52
Crucian carp (*C. auratus*)	BDE-15	Aqueous	0, 10, 100 µg/l; 50 d	BDE-15 accumulation in gill, liver 2 mono-brominated, 3 hydroxy metabolites	77
Zebrafish (Larv.)	BDE-209	Spiked sediment	12.5 mg/kg; 4 – 192 hpf	BDE-209 bioaccumulation	6
Rainbow trout (Juv.; *O. mykiss*)	DecaBDE (Dow FR- 300BA)	Diet	7.5 - 10 mg/kg bw/day; 16, 49, 120 d; 71 d depuration	Reductive debromination: hexa- to nonaBDE formation (liver, muscle); BDE-154 dominant BDE-209 uptake: 0.02–0.13% BDE-209 accumulation: 870 ± 219 ng/g ww (liver); 38 ± 14 ng/g ww (muscle)	59
Zebrafish (Juv.; *D. rerio*)	PentaBDE (DE-71)	Aqueous	0, 0.1, 1 mg/l; 4 wk	AhR-mediated effects linked to PBDD/F impurities; Weak induction CYP1A; no DR-CALUX response (purified DE-71)	78
Lake whitefish (Juv.; *C. clupeaformis*)	BDE-209	Diet	0, 0.1, 1, 2 µg/g; 30 d	Accumulation: BDE-209 + nonaBDEs (BDE-206, -207, -208) in liver	79
Zebrafish (Adu.)	BDE-28, -183, -209 (mix)	Diet	1 and 100 nmol/g ww food at 2% bw/day; 42 d with 14 d dep	Reductive debromination (high dose); 12 nmol of BDE-154/g ww fish; 3 nmol of BDE-149/g ww fish; < 2 nmol of BDE-153 g ww fish; Uptake: BDE-28 (100%) > BDE-183 (10%) > BDE-209 (< 1%)	57
Common sole (Juv.; *S. solea* L.)	BDE-28, -47, -99, -100, -153, -209 (mix)	Diet	82 - 184 ng/g ww food at 0.8% bw/day; 84 d, 149 d dep	Uptake efficiency: BDE-28 = 16%; BDE-47 = 15%; BDE-99 = 13%; BDE-100 = 14%; BDE-153 = 10%; BDE-209 = 1.4%. Reductive debromination: BDE-49; BDE-154; BDE-183; BDE-202; unk tetra-, penta-, hexaBDEs	56
Common sole (Juv.)	BDE-28, -47, -99, -100, -153, -209 (mix)	Diet	82 - 184 ng/g ww food at 0.8% bw/day; 84 d, 149 d dep	Oxidative metabolism: 6-OH-BDE-47; 4′-OH-BDE-49; 4′-OH-BDE-101; 4′-OH-BDE-103 No MeO metabolites detected	80
Common carp (Adu.)	BDE-99	Liver microsomes, cytosol	354 pmol; 1 - 250 µM; 90 min incub.	Reductive debromination (BDE-99 to BDE-47) more prevalent in liver microsomes than cytosol THs (rT3, T4) and iodoacetate inhibited debromination; role for deiodinase enzymes in reductive metabolism	81
Fathead minnow (Juv., *P. promelas*)	BDE-209	Diet	10 µg/g food at 5% bw/day; 28 d	BDE-209 bioaccumulation BDE-209 uptake efficiency = 5.8% Reductive debromination to penta-octaBDEs BDE-154 dominant metabolite BDE-101 lowest MW metabolite	61
Fathead minnow (Adu.)	BDE-209	Diet	95 ng/g ww food and 10 µg/g ww food at 3% bw/day; 28 d with 14 d dep	BDE-209 bioaccumulation Reductive debromination to penta – octaBDEs BDE-154 dominant metabolite BDE-101 lowest MW metabolite	62

dep = depuration; EROD = ethoxyresorufin-O-deethylase; dpf = days post fertilization; dph = days post hatch; dio = deiodinase; DR-CALUX = chemical-activated luciferase gene expression mediated by Ah-receptor activation; GI = gastrointestinal; HDT = highest dose tested; hpf = hours post fertilization; PBPK = physiologically based pharmacokinetic; UDPGT = uridine diphosphate glucuronosyl phosphate.

Table 1. PBDE toxicokinetics measured in teleost fish species (continued)

Species	Treatment	Route	Dose/Duration	Effects Observed	Ref
Atlantic cod (Juv., *G. morhua*)	BDE-47	Aqueous	5 µg/l; 21 d	Liver: ↓ mRNA transcripts encoding CYP1A, CYP2C33-like, CYP3C1-like, UDPGT No effects on antioxidant genes (GSH-Px, GR)	82
Rainbow trout Common carp Chinook salmon (*O. tschwatcha*) (Juv.)	BDE-28, -47, -49, -99, -100, -153, -154, -183, -203, -208, -209	Liver microsomes	1 µM; 24 h (hepta to BDE-209); 1 h (tri- to hexaBDEs)	Reductive debromination of BDE-99, -153, -183, -203, -208, -209 Carp: *meta*-position debrom dominated Salmonids: *meta*- and *para*-position debrom No metabolism of PBDEs lacking *meta*-substituted Br (BDE-28, -47, -100)	83
Rainbow trout Common carp (Juv.)	BDE-209	Diet, in vitro	940 ng/g ww food, 1% bw/day; 5 mo; 15 pmol/mg protein; 1, 24 h (microsomes)	Reductive debromination (trout): Formation BDE-207, -208, -188, -201, -202, unk. octa-heptaBDEs BDE-209 uptake (trout): 3.2%; liver > serum > intestine > carcass (lipid-normalized) In vitro: Formation of octa – nonaBDEs (trout), hexa - octaBDEs (carp)	60
Common carp (Juv.)	BDE-209	Diet	940 ng/day-fish; 60 d w/40 d dep	Reductive debromination: Formation of BDE-154, BDE-155, unknown hexa- to octaBDEs No BDE-209 bioaccumulation	25
Common carp (Juv.)	BDE-99, BDE-183	Diet	400 ng/day-fish (BDE-99); 100 ng/day-fish (BDE-183); 62 d w/37 d dep	Reductive debromination (GI tract): BDE-99 ⇢ BDE-47 BDE-183 ⇢ BDE-154, unk hexaBDE Uptake: BDE-99 = 9.5%; BDE-183 = 17%	58
Common carp (Juv.)	BDE-28, -47, -99, -153 (mix)	Diet	470 ng/day-fish; 60 d w/40 d dep	BDE-47 accumulation, high assimilation No BDE-99 bioaccumulation; No hydroxy metabolites detected	84
Japanese medaka (Adu.; *O. latipes*)	6-OH-BDE-47, 6-MeO-BDE-47, BDE-47	Maternal	21, 8, 0.9 µg/g dw food at 2% bw/day; 14 d	No OH-, MeO-BDEs in BDE-47 treated fish; In vivo and in vitro conversion of 6-OH-BDE-47 to 6-MeO-BDE-47 (and vice-versa) Maternal transfer to eggs	85
Common carp (Juv.)	Penta and DecaBDE mixtures	Diet	100, 120, 150 µg/day/fish; 20 d	Reductive debromination facilitated by at least one meta- or para- doubly flanked Br 11 OH-BDEs measured in serum of pentaBDE exposed fish; No OH-BDEs in decaBDE exposed fish	86
Chinese sturgeon (Adu; *A. sinensis*)	BDE-209	Field collected	Liver microsomes; PBPK modeling	Reductive debromination; Formation of BDE-126, -154, -188, -202, -204, -197; Low partition coefficients from blood to tissues lead to higher bioaccumulation of hepta to BDE-209 in absorbing tissues	63

dep = depuration; EROD = ethoxyresorufin-O-deethylase; dpf = days post fertilization; dph = days post hatch; dio = deiodinase; DR-CALUX = chemical-activated luciferase gene expression mediated by Ah-receptor activation; GI = gastrointestinal; HDT = highest dose tested; hpf = hours post fertilization; PBPK = physiologically based pharmacokinetic; UDPGT = uridine diphosphate glucuronosyl phosphate.

not been found to be involved in the reductive debromination of BDE-99 to BDE-49 in Chinook salmon[76] or of BDE-99 to BDE-47 in common carp,[74,81] suggesting that they may not play an important role in PBDE debromination in fish. This finding has been subsequently confirmed in negative results from in vitro testing with liver microsomes from Chinook salmon, rainbow trout, and common carp incubated with several PBDE congeners[83] and in juvenile fathead minnows exposed in vivo to BDE-209.[61] Taken together, despite these lines of indirect evidence, additional work is needed to better understand the underlying enzymes catalyzing the reductive debromination of PBDEs in fishes, and the potential role of Dio enzymes and/or other possible reductases that have yet to be described.

Mixed Evidence of PBDE Oxidative Metabolism in Fish

Laboratory studies in fish have presented mixed results concerning the formation of hydroxylated PBDE (OH-BDE) metabolites. For instance, no OH-BDEs were detected in the serum of common carp receiving dietary exposures to a mixture of BDE-28, -47, -99, and -153.[84] Likewise, OH-BDEs were not detected in liver microsomes from common carp, rainbow trout, or Chinook salmon incubated with various PBDEs, including BDE-209.[76,83] In contrast, 11 OH-BDEs were reported in the serum of juvenile common carp exposed to the PentaBDE mixture with no OH-BDEs in DecaBDE exposed fish.[86] Another BDE-209 study, however, reported substantial OH-BDE and methoxy PBDE (MeO-BDE) formation in trout.[93] OH-BDE metabolites were

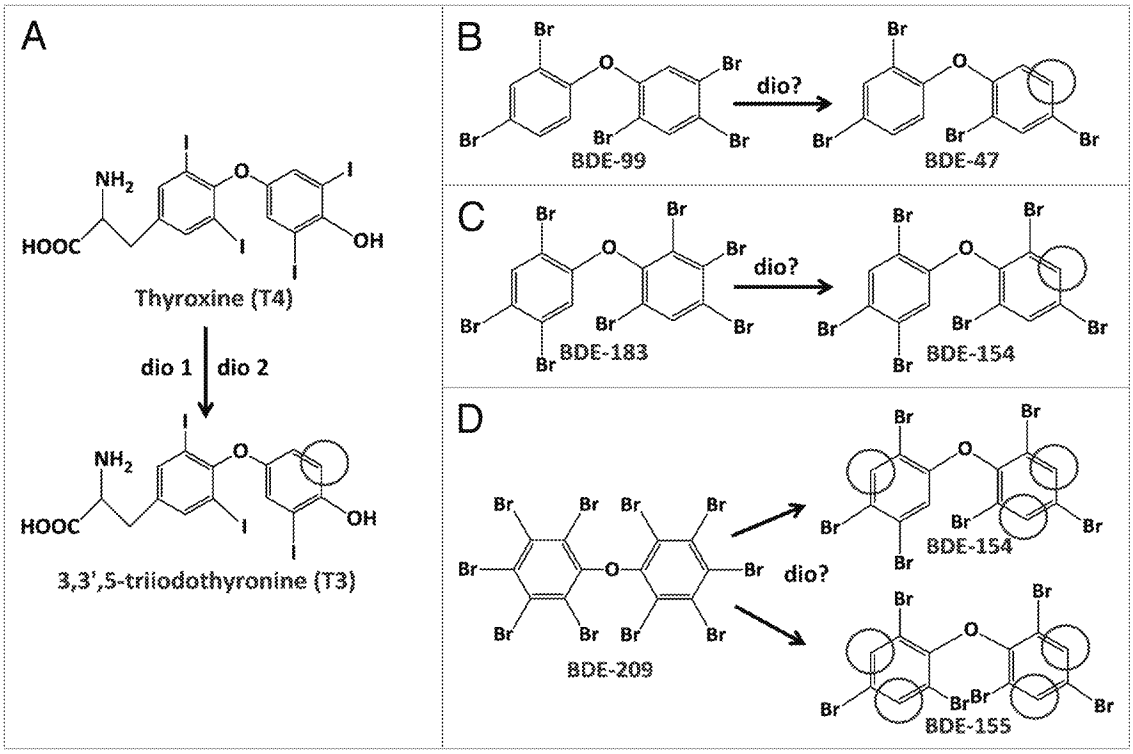

Figure 2. Outer-ring deiodination of thyroxine (A) catalyzed by Dio 1 and 2 enzymes resulting in meta-cleavage of iodine from T4. Reductive metabolites and meta-position debromination measured in common carp (*C. carpio*) exposed orally to (B) BDE-99, (C) BDE-183, and (D) BDE-209.[25,58,60]

also reported in juvenile common sole (*S. solea* L.) exposed to a mixture of congeners (BDE-28, -47, -99, -100, -153, -209).[80] In studies showing positive OH-BDE results, the gas chromatography-mass spectrometry (GC/MS) injection techniques used in the PBDE analyses were either not specified[86] or employed split/splitless injection.[80,93] GC/MS splitless injection techniques for PBDE analysis can lead to thermal degradation of parent PBDEs and the formation of byproducts in the GC/MS inlet that may confound identification of MeO-BDEs (GC/MS derivatives of OH-BDE metabolites).[94] Although potential analytical confounders were not addressed, if oxidative metabolism occurs in fish, it appears to be a minor metabolic pathway compared with reductive debromination.

In vivo and in vitro studies in fish have shown both weak induction[78,95] and inhibition[82,96] of ethoxyresorufin-O-deethylase (EROD) activity (biomarker of CYP1A and aryl hydrocarbon (AhR) induction) upon exposure to individual PBDE congeners. However, a larger number of PBDE studies in teleost fish have shown no AhR/CYP1A activation.[75,89,97,98] Thus, it appears that PBDEs operate predominantly through non-dioxin, AhR independent toxicity mechanisms and are not metabolized by CYP1A. Conversely, the PentaBDE commercial mixture contains small amounts of polybrominated dibenzo-*p*-dioxins/dibenzofurans (PBDDs/PBDFs) that are trace byproducts formed by thermal stress during production and that activate the AhR. The reasons for the disparate results in the literature are not clear but may be attributable to variations in the purity of formulations tested.

While mammalian studies support PBDE oxidative metabolism by CYP2B and CYP3A catalyzed by CAR/PXR inductions, similar pathways have not been observed in fishes. The expression of CYP2B in fish and the regulatory mechanisms involved in its induction are still unclear. In mammals, phenobarbital (PB) and ortho-substituted halogenated aromatics (e.g., ortho-chlorine-substituted PCBs) are strong inducers of CYP2B through activation of CAR.[99] In teleost fish, however, the induction of CYP2B in the presence of PB-type inducers has not been observed.[100] Thus, there appear to be important functional differences between piscovorous and mammalian CAR/CYP2B that may play a role in its lack of induction in PBDE-exposed fish, although this has not been examined. With regard to other AhR-independent mechanisms, the function and tissue distribution of enzymes in the CYP3 gene family are not well characterized in fish, but the CYP3A isoforms appear highly versatile with broad substrate affinities.[101]

PBDE Conjugation in Fish

There has been little research to date to examine the role of UDPGTs and SULTS in the metabolism of PBDEs in fishes, although these enzymes are important catalytic drivers of Phase II metabolism in fishes.[100] The UDPGTs catalyze the glucuronidation of an array of endogenous and exogenous substrates to more polar, water-soluble compounds for elimination. The SULTs catalyze the transfer of the sulfonate from

Table 2. Whole-body elimination half-lives ($t_{1/2}$) of environmentally relevant PBDEs measured and estimated in humans, laboratory rodents, and teleost fish species

PBDE Congener	Teleost Fish half-life (days)	Humans half-life	Rodents half-life (days)
BDE-47	18–42 (Common carp)[84] 173–519 (Lake trout)[89] 12 (Japanese medaka)[108]	1.5–2.5 y[109]	25c (mouse)[55] 19–30e (rat, tetraBDE)[110]
BDE-99	173–519 (Lake trout)[89]	1.8–3.4 y[109]	6d (rat)[91]
BDE-100	46–80 (Lake trout)[89]	1.3–1.8 y[109]	42–52e (rat, pentaBDE)[110]
BDE-153	4–23 (Common carp)[84] 154–308 (Lake trout)[89]	3.6–12.4 y[109]	50–105e (rat, hexaBDE)[110]
BDE-154	17–53 (Common carp)[84] 70–208 (Lake trout)[89]	2.3–4.3 y[109]	Unknown
BDE-183	173–519 (Lake trout)[89] 10–15 (Zebrafish)[57]	94 d[111]	Unknown
BDE-209	21–34 (Lake trout)[89] 6.5 (Zebrafish)[57]	15 d[111]	2.5, 8.6 (rat)[112,113]

3′-phosphoadenosine-5′-phosphosulfate (PAPS) to hydroxylated and amine substituents on numerous exogenous and endogenous substrates to facilitate elimination. In zebrafish, several UDPGT[102] and SULT genes[103,104] have been characterized with prototypical substrates such as bilirubin, TH, estradiol (E2), testosterone (T), and phenolic contaminants. As many as 10 different UDPGT isoforms have been identified in zebrafish, with nucleotide similarities to some mammalian UGT1 and UGT2 gene families.[105] Two studies have shown a decrease in the relative mRNA abundances of genes encoding *UDPGT1ab* in zebrafish larvae exposed to BDE-209[106] and *UGT1* in juvenile Atlantic cod (*G. morhua*) exposed to BDE-47.[82] This decline may be a response to reduced TH levels as UDPGTs are involved in the metabolism of THs.

PBDE Elimination

Table 2 provides whole body elimination half-lives ($t_{1/2}$) reported in fish for PBDEs frequently detected in the environment and biota, with inclusion of data in humans and rodents for comparison. In rodents, the major route of PBDE elimination is by the fecal route with low levels of excretion in the urine and bile depending on the PBDE congener.[88,107] In fish, routes of PBDE elimination have not been targeted specifically but the early studies in pike suggest that biliary and fecal excretion also occurs.[52] Some reports estimate apparent half-lives in humans for the tetra - hexaBDEs that are substantially longer than those reported in rodents and some fish. Conversely, some data in rodents suggest relatively short half-lives (e.g., BDE-99) that are incongruous with the human and fish data. Thus, there continues to be uncertainty about PBDE elimination half-lives in fish and other biota with substantial species variability apparent that appears related to differential metabolism.

Fish Thyroid System

To discuss PBDE-related TH disruption, it is informative to broadly highlight current knowledge of the structure and function of the fish thyroid with some comparison to the mammalian thyroid. The vertebrate thyroid is well-conserved across taxa, and chemical effects in lower level vertebrates like fish can reveal mechanisms of thyroid dysregulation in higher level species. THs are key regulators of vertebrate development, endothermic basal metabolism, and organ system physiology. The importance of TH in brain and somatic development is well established, and small changes in maternal or fetal TH can cause severe motor skill deficiencies and irreversible cognitive impairments.[114] Recent attention has focused on the permissive role of THs in regulating physiological processes in adults, including neurological plasticity, mood, cognition, and reproduction.[90,115] In fish, THs are important mediators of many physiological, developmental, and behavioral processes, including growth and metamorphic transitioning,[116,117] osmoregulation,[118] olfactory imprinting,[119] interrenal regulation,[120] otolith formation,[121] and reproduction,[90,122] often acting in concert with other hormones.[123,124]

Structure and Function of Fish Thyroid

The general architecture of the thyroid appears to be similar across vertebrates whereby circulating levels of TH are tightly controlled by both a centrally operating hypothalamic-pituitary-thyroid (HPT) axis and in peripheral tissues through the activity of Dio enzymes, among other dynamically operating regulatory processes (**Fig. 3**). The functional unit of the central HPT is the thyroid follicle where the THs T4 and 3,5,3′-triiodothyronine (T3) are synthesized and secreted into circulation. However, there continue to be gaps in our understanding of the fish thyroid in comparison to the more thoroughly studied mammalian and amphibian thyroid systems. Typically in teleost fish,

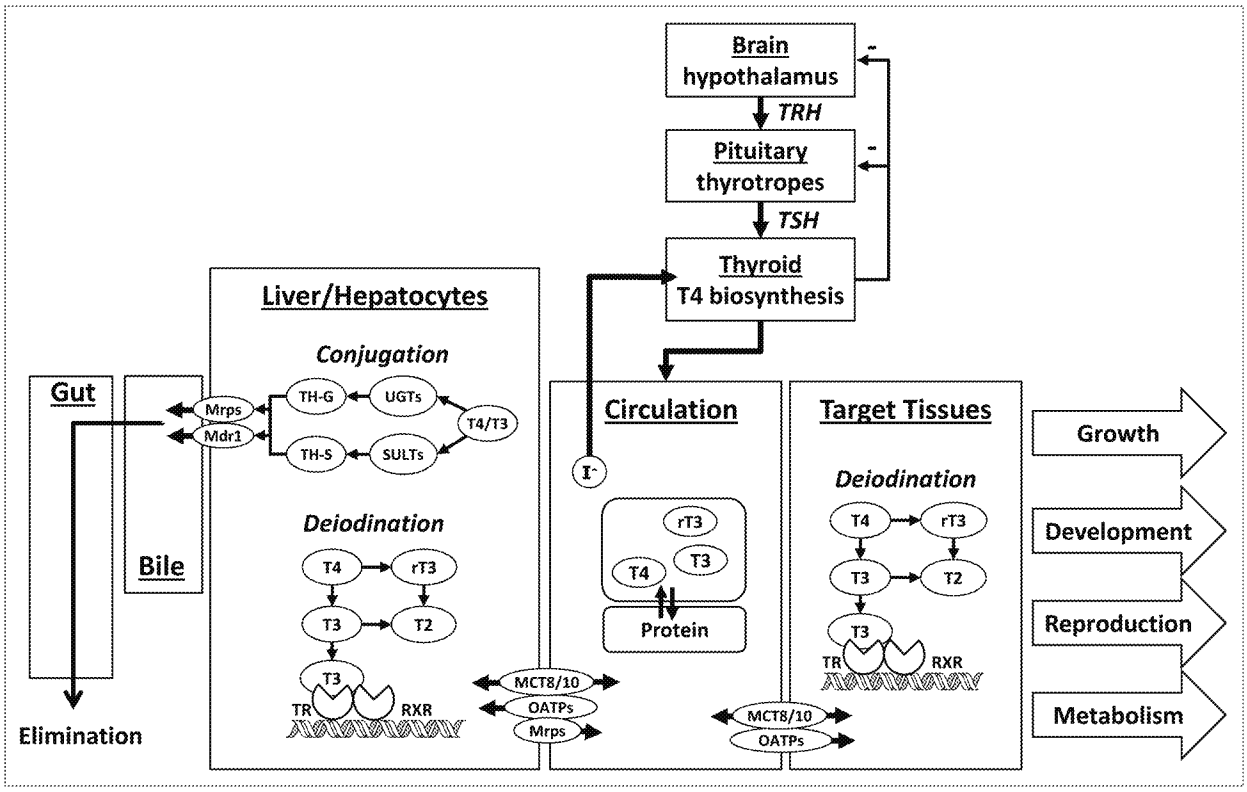

Figure 3. Overview of TH regulation and signaling in teleost fishes. TRH = thyrotropin releasing hormone; TSH = thyroid stimulating hormone; T4 = thyroxine; T3 = 3,3′,5-triiodothyronine; rT3 = 3,3′,5′-reverse T3; T2 = 3,3′-diiodothyronine; UGT = uridine diphosphate glucuronosyl transferase; SULT = sulfotransferase; TH-G = glucuronidated thyroid hormone; TH-S = sulfated thyroid hormone; Mrp = multidrug resistance associated protein; Mdr1 = multidrug resistance protein 1 or P-glycoproteins; MCT = monocarboxylate transporter; OATP = organic anion transport polypeptide; TR = thyroid hormone receptor; RXR = retinoic x receptor.

thyroid follicles are found dispersed predominantly in the ventral pharyngeal region, rather than being organized in a compact lobular gland as seen in higher vertebrates. One important differentiating feature of the fish thyroid may relate to how THs are produced and regulated. In fish, T4 may be the primary, possibly only TH produced in the thyroid where it is under negative feedback control by the HPT axis. The production of T3 in fish, in contrast, is thought to be under exclusive control of peripheral tissues, although this has not been studied recently or beyond Salmonid fishes.[125] In some contrast, the mammalian thyroid gland produces both T4 and to a lesser extent T3 under negative feedback control by the central HPT. Thus, while extrathyroidal regulation of T3 is important in all vertebrates, including for instance in the brains of developing mammals, in fish the formation and regulation of T3 may be dominated by local control in response to the needs of individual tissues rather than by T4 availability and through the central HPT axis. THs circulate in plasma bound to TH binding proteins, including thyroid binding globulin (TBG), transthyretin (TTR), and albumin. In humans, the primary transporter of TH is TBG while in rodents it is TTR.[126] Less is known about the dominant transporters in fish although it has recently been shown that TTR may bind TH in some species.[127,128] Most TH in fish circulation is bound to protein with only a small amount (< 1%) thought to be free and available for uptake into cells. As demonstrated in rodents, the cellular transport of THs in fish is mediated largely by membrane bound transporters, including the high affinity monocarboxylate transporter 8 (MCT8) and organic anion transporter polypeptides (e.g., OATP1c1), among others.[129-132]

Peripheral TH Regulation and Signaling in Fish

Once in the cell, T4 can be deiodinated to the active T3 hormone or inactivated to 3,3′,5′-triiodothyronine (rT3) or 3,3′-diiodothyronine (T2) (Fig. 4). Dio 1 and Dio 2 enzymes in vertebrates may catalyze T4-outer ring deiodination (ORD) to produce the active T3 hormone, while Dio 1 and Dio 3 catalyze T4-inner ring deiodination (IRD) to inactive rT3. Thus, Dio 1 can be involved in both ORD and IRD. In addition, T3-IRD and rT3-ORD can metabolize hormone to T2. THs in vertebrates are further conjugated in the liver to glucuronides or sulfates catalyzed by UDPGTs and SULTs, respectively, and excreted through the bile and urine. The transcriptional activity of T3 is mediated by complexing with nuclear thyroid receptors (TRs) that bind to TH response elements (TREs) to induce the expression of TH responsive genes.[133] Increased attention is also focusing on nongenomic pathways of THs signaling by integrin mediated signaling and kinase activation.[134,135]

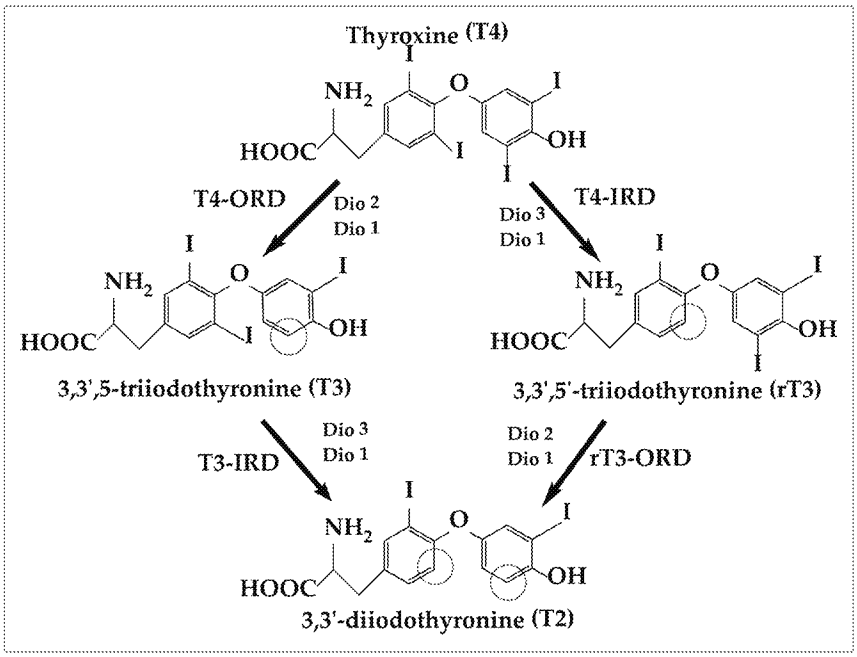

Figure 4. Pathways of outer and inner ring deiodination of thyroid hormones in extrathyroidal peripheral tissues of vertebrates.

While Dio enzymes in fish and mammals are believed to share many functional features, relative tissue distributions and activity vary, which may have implications for their role in TH regulation.[136,137] For instance, Dio 1 has been localized to the kidneys and liver of fish and mammals (as well as in the mammalian thyroid gland).[138] However, hepatic T4-ORD activity is thought to be catalyzed mostly by Dio 1 in mammals and Dio 2 in fish.[136,139] One notable exception to this peripheral TH production may occur in the teleost fish brain with its relatively low T4-ORD activity. Early studies that measured deiodination in rainbow trout attributed reduced brain T4-ORD in fish to absent or negligible Dio 2 expression.[140] However, more recent studies using quantitative PCR have localized mRNA expression of *dio2* genes in the fish brain, although at comparatively lower levels than in other tissues.[62,141,142] Limited evidence also suggests that the transcriptional response of Dio 2 in the fish brain may be more sensitive to systemic TH changes than Dio 2 in the liver.[141] Likewise, in mammals, Dio 2 has demonstrated substantial physiological plasticity in the brain with a short half-life of ~40 min suggesting that it might also be an important regulator of intracellular T3 in these tissues.[143]

PBDE Thyroid Disruption in Fish

Because PBDEs, particularly OH-BDE metabolites, are similar in structure to THs, concerns have been raised about their effects on thyroid system functioning in both mammalian and non-mammalian vertebrates.[50] In fish, PBDEs have been shown to perturb the thyroid system at several points along the central HPT and in extrathyroidal tissues with most study to date focused on the PentaBDE mixture, BDE-47, and BDE-209. Many of these laboratory studies in fish have shown declines in circulating levels of THs and altered TH signaling in some species exposed to these PBDE congeners and mixtures. Table 3 provides a summary of studies in fish conducted to date. Similar to results in fish, in vivo rodent studies and cell-based assays have reported PBDE-induced disruption of thyroid homeostasis, including declines in circulating THs[144-146]; altered expression and activity of TH metabolizing enzymes,[147,148] and competitive binding with plasma transporters.[149] Human epidemiology studies have shown associations between altered plasma concentrations of THs in adults and PBDE levels in serum and dust.[150-152]

BDE-47, PentaBDE, and OH-BDE Thyroid Dysfunction

In one of the earlier studies in fish, depressed levels of circulating free T4 and T3 were measured in the plasma of lake trout (*S. namaycush*) dosed through the diet with a mixture of 13 PBDE congeners (e.g., BDE-28, -47, -99, -100, -153, -154, -209) at 2.5 ng/g and 25 ng/g per congener for 56 d.[89] In fathead minnows, dietary exposures to BDE-47 at 2.4 and 12.3 µg/breeding pair-day for 21 d elicited depressed total T4 (TT4) but not total T3 (TT3) that was accompanied by elevated mRNA transcripts encoding TSHβ in the pituitary of low dose fish.[154] In addition, elevated and reduced levels of mRNA transcripts encoding *TR*α and *TR*β, respectively, were detected in the brain but not the liver, suggesting that the adult fish brain may be a sensitive target of BDE-47.[154] These results in minnows and trout are consistent with observations in European flounder (*P. flesus*) whereby declines in circulating TT4 with no change in TT3 were detected after a 101 d dietary exposure to the PentaBDE commercial mixture purified to remove polybrominated dibenzo-*p*-dioxins/dibenzofurans (PBDDs/Fs).[96]

Reductions in whole fish T4 have also been measured in zebrafish larvae subjected to waterborne exposures of the PentaBDE commercial mixture.[157] In some divergence, however, this same research group measured elevated whole fish T4 and T3 in zebrafish offspring exposed to PentaBDE at the same dose as their previous study (Yu et al., 2010) but by a different exposure route (maternal) and longer duration (5 mo). The opposing effects of PentaBDE on TH levels that were measured in the Yu et al. (2010–11) zebrafish work demonstrate potentially important differences in PBDE impacts on vertebrate thyroid signaling that may be influenced by the pathway and duration of exposure (e.g., aqueous, short-term vs. maternal, chronic). BDE-209 and the PentaBDE mixture also have been found to enhance the relative mRNA expression of genes encoding *dio1* and *dio2* in zebrafish larvaeas well as thyroidal genes encoding the sodium iodide symporter (NIS), thyroglobulin (TG) and other transcription factors regulating NIS and TG expression (Nkx2.1a, Pax8).[106,157]

Table 3. PBDE effects on thyroid endocrine systems of teleost fish

Species	Treatment	Route	Dose/Duration	Effects Observed	Ref
Zebrafish (Larv.)	BDE-209	Aqueous	0, 0.08, 0.38, 1.92 mg/l; 14 dpf	↑ mRNA transcripts for CRH, TSHβ, Pax8, Nkx2.1, NIS, Tg, dio1, dio2, trα, trβ ↓ TTR mRNA transcripts; ↑ T3 (0.38, 1.92 mg/l), ¯T4 (1.92 mg/l)	106
Zebrafish (Juv.)	BDE-47	Diet	100 ng/g; 0.5–1 mg food/ fish-d; 20–60 dpf	No change in TTR, dio1, TSHβ mRNA transcripts	95
Zebrafish (Larv.)	6-OH-BDE-47	Aqueous/WISH	1, 10, 100 nM; 4 – 22 hpf	↑ dio1 mRNA in brain periventricular zone; ↑ dio 3 in pronephric duct	153
Rainbow trout (*O. mykiss* Juv.)	BDE-209	IP Injection	50 – 1000 ng/g bw/day; 21 d	↑ TT4 (1000 ng/g bw); ↓ FT3 (all doses); ↓ FT4 (100, 200, 500 ng/g bw)	93
European flounder (*P. flesus* Adu.); Zebrafish (Juv.)	PentaBDE (DE-71 purified of PBDD/Fs)	Spiked sediments, Diet	0+0.014 -700+14000 µg/g TOC + µg/g lipid; 101 d (flounder) 0–500 µg/l; 30 d (zebrafish)	↓ plasma TT4 (flounder) ↑ plasma TH, ↓ larval survival; (zebrafish)	96
Fathead minnow (Adu. breeding pairs)	BDE-47	Diet	2.4 µg/pair/ day; 12.3 µg/pair/ day; 21 d	↓ TT4 (no change in TT3); ↑ mRNA transcripts for TSHβ (low dose), trα (female brains); ↓ trβ mRNA transcripts (both sexes, brains)	154
Chinese rare minnow (*G. rarus*; Adu., Larv.)	BDE-209	Aqueous	0 – 10 µg/l; 21 d	↑ dio2, NIS mRNA transcripts (larvae) ¯trα, dio2, NIS mRNA transcripts (adults)	155
Gilthead sea bream (*S. aurata*; Adu.)	PBDEs, 6-OH-BDE47	In vitro	0–10 µM; 2 h	BDE-28, 49, -47, -99 potent inhibitors of ^{125}I-T3 binding to TTR; IC_{50}s < < T3, T4 6-OH-BDE-47 moderate inhibitor; IC_{50}s > T3,T4	156
Fathead minnow (Juv.)	BDE-209	Diet	10 µg/g food at 5% bw/day; 28 d with 14 d dep	↓ Dio activity (T4-ORD and T4-IRD) by 74% Thyroid histology: Over-stimulation, injury (thickened follicular epithelium, irregular follicle outlines, colloid depletions, ↑ inflammatory cells) Liver alterations: vacuolated hepatocytes	61
Fathead minnow (Adu. males)	BDE-209	Diet	95 ng/g ww food and 10 µg/g ww food at 3% bw/day; 28 d with 14 d dep	↓ TT4 (53%) and TT3 (46%) at low dose ↓ TT4 (59%) and TT3 (62%) at high dose ↓ brain Dio activity ~65% ↑ mRNA transcripts for dio1, dio2, trα, trβ (↓ in liver), mct8, oatp1c1, oatp2a1(↓ in liver) in brain and liver	62
Lake trout (*S. namaycush*; Juv.)	13 PBDE congener mix	Diet	0, 2.5, 25 ng/g dw food at 1.5% bw/day; 56 d; 112 d depuration	↓ FT4 (low, high dose); ↓FT3 (low dose only)	89
Zebrafish (Larv.)	PentaBDE (DE-71)	Aqueous	1, 3, 10 µg/l; 14 d	↓ whole fish T4 (T3 not measured) ↑ mRNA transcripts for CRH, TSHβ, NIS, Tg, Pax8, Nkx2.1, dio1, dio2; ¯TTR mRNA transcripts	157
Zebrafish (Adu., offspring)	PentaBDE (DE-71)	Aqueous	1, 3, 10 µg/l (parents and offspring) + (parent alone); 5 min to sexual maturation (parents), 5 and 10 dph (offspring)	↑ plasma TT4 (no Δ TT3) (parents, ELISA); ↓ mRNA transcripts for CRH, TSHβ (parent brain) ↑ whole fish T4, T3; altered HPT axis mRNA transcripts (offspring w/w/o DE-71 exposure)	158

BTEB = basic transcription element-binding protein; CRBP = cellular retinal binding protein; CRH = corticotrophin releasing hormone; CSF = cerebral spinal fluid; dep = depuration; dpf = days post fertilization; dph = days post hatch; dio = iodothyronine deiodinase; FT3 = free triiodothyronine; FT4 = free thyroxine; HDT = highest dose tested; hpf = hours post fertilization; HPT = hypothalamic-pituitary-thyroid; MBP = myelin basic protein; PBDD/f = polybrominated dibenzo-p-dioxins/dibenzofurans; NIS = sodium/iodide symporter; Tg = thyroglobulin; TH = thyroid hormone; TOC = total organic carbon; TR = thyroid receptor; TSH = thyroid stimulating hormone; TT4 = Total thyroxine; TTR = transthyretin; WISH = whole mount in situ hybridization.

There has been only limited thyroid-related study in fish to date focused on the OH-BDEs. Recent work in our laboratory using whole mount in situ hybridization (WISH) measured significantly upregulated mRNA expression of *dio1*, but not *dio2* or *dio3*, in the periventricular brains of 22 hpf embryos exposed to the hydroxylated metabolite 6-OH-BDE-47. An increase in *dio3* mRNA expression was also detected in the pronephric duct, which is the earliest form of the kidney in vertebrates and constitutes the central component of the excretory system. Thus, this study demonstrated that effects of 6-OH-BDE-47 on the developing zebrafish thyroid may elicit localized and age-specific transcriptional responses that then potentially contribute to downstream effects on neurological, renal, and reproductive development. To better understand the implications of these findings, additional data are needed to clarify the cellular and tissue distribution of Dios during ontogeny of the fish brain. In the mammalian brain, Dio 2 is expressed in glial cells (astrocytes, tanycytes), which could play a role in transporting and maintaining T3 supplies to neurons.[159]

BDE-209 Thyroid Dysfunction

BDE-209 reduced circulating levels of free T4 and T3 in early life-stages of rainbow trout[93] with declines in whole fish T4 also reported in zebrafish larvae exposed aqueously to BDE-209.[106] In addition, an increase in *dio2* mRNA transcripts have been measured in the larvae of Chinese rare minnows exposed to BDE-209 with a decrease in *dio2* transcripts measured in the brains of adults minnows.[155] Recently published data of ours has shown TH regulation and signaling in juvenile and adult fathead minnows to be disrupted by low dose exposures to BDE-209. Specifically, in juvenile fathead minnows, compared with vehicle controls, the activity of Dio enzymes (T4-ORD and T4-IRD) declined by ~74% in fish dosed with 9.8 μg/g ww food at 3% bw/day for 28 d.[61] This extrathyroidal perturbation was accompanied by evidence of thyroid follicle hypertrophy indicative of over-stimulation and injury. In adult male fathead minnows, BDE-209 caused a > 53% and > 46% decline in circulating total T4 and T3, respectively, upon a 28-d exposure to low doses of BDE-209 at ~3 and 300 ng/g bw-day.[62] Depressed levels of circulating THs were accompanied by a 65% decline in Dio activity (T4-ORD) in the brains of treated fish at both BDE-209 doses tested. This hypothyroid response in BDE-209 exposed minnows was accompanied by possible localized compensatory signaling, including increased T4-ORD activity in the liver and transient, tissue-specific upregulation of genes encoding several important thyroidal proteins (Table 4). However, similar to results in minnows exposed to BDE-47,[154] this study suggested that the fish brain may be particularly sensitive to BDE-209 based on severe reductions in brain Dio activity (T4-ORD) and potentially muted adaptive responses of the brain to reduced TH levels. Consistent with observations in the brains of adult fish, data collected in developing rodents suggest weak adaptive responses of the brains of younger mammals to TH insufficiency caused by low level chemical exposures.[160-162] Additional work is needed to better understand how tissues, especially the brains, of developing and adult animals adapt or not to contaminant-induced TH insufficiency and whether this ameliorates downstream apical endpoints.

Mechanisms of PBDE Thyroid Disruption

The vertebrate thyroid system maintains normal physiological functioning by responding to endogenous and exogenous perturbations with changes in TH production from the thyroid and through changes in the capacity and sensitivity of peripheral tissues. Such integrated compensatory responses at the central HPT and in peripheral target tissues make it challenging to evaluate mechanisms of action for thyroid disruptors like PBDEs. Nonetheless, several mechanisms appear to play a role in the thyroid perturbations measured in fish (and other vertebrates) exposed to PBDEs including: interference with Dio enzyme activity/expression; enhanced metabolism and elimination of THs; altered expression and activity of plasma transporters and membrane bound transporters; and altered genomic signaling.

Interference with Dio Enzymes

One mechanism that might be contributing to thyroid perturbations in fish and other vertebrates could involve PBDEs interfering with the expression and activity of Dio enzymes. PBDEs may be acting as competitive substrates for Dio enzymes or otherwise altering the expression and activity of these enzymes. As discussed, altered mRNA expression and enzymatic activity of some Dio isoforms has been observed in fish exposed to BDE-209[61,62,155] and 6-OH-BDE-47,[153] as well as in rodents exposed to PentaBDE[148] and human microsomes incubated with 5′-OH-BDE-99 and 2,4,6-TBP.[147] However, it remains unclear whether PBDEs (or OH-BDEs) can bind directly to Dio enzymes or whether they may elicit other allosteric effects that affect the capacity of Dios to mediate TH regulation.

Induction of TH Metabolizing Enzymes

Another hypothesis for PBDE-induced thyroid disruption is that PBDE detoxification responses may induce the expression and/or activity of TH catabolizing enzymes. In some support of this hypothesis, studies have measured increased mRNA expression of TH-conjugating UDPGT and SULT enzymes in rodents exposed to BDE-47[145] and the PentaBDE commercial mixture.[148,163] In these studies, declines in circulating levels of T4 from PBDEs were linked to enhanced glucuronidation associated with UDPGT inductions.[148,163,164] Other studies, however, have shown little to no change in UDPGT levels in rodents following exposure to PBDEs despite decreased T4 levels in circulation,[145,165] although increased mRNA expression of UDPGT transcripts has been observed in some of this work.[145] In contrast to the rodent data, limited evidence in fish has shown declines in

Table 4. Relative expression of genes encoding deiodinase (*dio*) enzymes, nuclear thyroid receptors (*tr*), monocarboxylate transporters (*mct*), and organic anion transporter polypeptides (*oatp*) in brains and livers of adult male fathead minnows exposed orally to BDE-209 and the positive control 6-propyl-2-thiouracil (PTU) for 28 d with a 14-d depuration.[62]*

Gene target	Treatment	Day 14		Day 28		Day 42	
		Brain	Liver	Brain	Liver	Brain	Liver
dio1	BDE-209 Low Dose	—	—	↑	—	—	↑
	BDE-209 High Dose	—	—	↑	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
dio2	BDE-209 Low Dose	↑	↑↑	—	—	—	
	BDE-209 High Dose	↑↑	↑	—	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
trα	BDE-209 Low Dose	—	—	—	—	↑	—
	BDE-209 High Dose	↑↑↑	—	↑↑	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—
trβ	BDE-209 Low Dose	—	↑	—	—	—	—
	BDE-209 High Dose	—	—	—	↓	—	—
	PTU Pos Ctrl	—	↑	—	—	—	—
mct8	BDE-209 Low Dose	↑	↑	—	—	↑↑	—
	BDE-209 High Dose	↑↑↑	—	—	—	—	—
	PTU Pos Ctrl	↑	—	—	—	—	—
oatp1c1	BDE-209 Low Dose	—	↑	—	—	—	—
	BDE-209 High Dose	—	↑	—	—	—	—
	PTU Pos Ctrl	—	↑	—	—	—	—
oatp2a1	BDE-209 Low Dose	—	—	—	—	—	—
	BDE-209 High Dose	↑↑	↓	—	—	—	—
	PTU Pos Ctrl	—	—	—	—	—	—

*Relative mRNA transcript abundances of genes not affected by BDE-209 or PTU: *dio3, mct10, oatp1f1, oatp1f2, oatp2b1,oatp3a1, oatp4a,* and *oatp5a1.* Statistical significance evaluated within sampling day with a one-way ANOVA and Tukey's test; one arrow = $P < 0.05$; two arrows = $P < 0.01$; three arrows = $P < 0.005$.

mRNA transcript abundances of some UDPGT isoforms suggesting that PBDEs may be acting as TH mimics that then downregulate the expression of these TH metabolizing enzymes in PBDE exposed fish.[82,106]

Altered Expression/Activity of Plasma and Cellular Transporters

Few studies have explored the role of plasma and membrane bound transporters in PBDE metabolic detoxification pathways and in contributing to or ameliorating PBDE effects on TH signaling.[166] OH-BDE metabolites produced in rat liver microsomes enriched with CYP2b (i.e., PB-induced) have been found to compete with THs for binding to the plasma transport protein TTR, potentially leading to greater elimination of TH and hypothyroidism.[149,167] Likewise in a recombinant sea bream TTR assay, several parent PBDEs (BDE-28, -49, -47, -99) were shown to be potent inhibitors of T3 binding to TTR, suggesting competitive interferences, while 6-OH-BDE-47 had less affinity for sea bream TTR than T3 or T4.[156] Other studies have also used biosensor screening methods to show that the OH-BDEs may bind to TTR and TBG with high potency.[166] More recently, newly designed fluorescent probes and competitive binding assays have shown that the binding of OH-BDEs with TBG and TTR increases with bromine number and OH position (i.e., 3-*meta* OH).[168]

In addition to plasma transport, a few studies have explored PBDE effects on cell membrane bound transporters of TH. Our recently published data in fathead minnows measured upregulated mRNA transcripts encoding *mct8* and *oatp1c1* in the brains and livers of fish exposed to BDE-209 (Table 4).[62] As observed with dio transcription, upregulated mRNA expression patterns of these transporters in minnows exposed to BDE-209 may be indicative of additional compensatory responses to hypothyroidism as *mct8* and *oatp1c1* are specific and active transporter of THs in fish[129,169] and mammals.[170,171] Only a limited number of the OATP transporters have been characterized in vertebrates with recent work in zebrafish[131] and fathead minnows[169] to clarify their tissue distribution and function. Some OATPs have been found to be potentially important PBDE transporters. BDE-47, -99, and -153 have been shown to be effective substrates for human OATP1B1, OATP1B3, and OATP2B1 expressed in Chinese hamster ovary (CHO) cells.[172] OATP1B1 and OATP1B3 were found to transport BDE-47 with the highest affinity, while OATP2B1

was found to transport all three tested congeners with similar affinities. Using human embryonic kidney cells transiently expressing mouse hepatic OATPs, this same research group also measured that OATP1a4, OATP1b2, and OATP2b1 were able to bind and transport BDE-47, -99, and -153.[173] Consistent with these results, upregulated mRNA expression of genes encoding OATP1a4, which transports THs, bile acids, and xenobiotics, was also detected in young rats exposed to PentaBDE.[148] PBDEs have also been shown to affect the Phase III hepatic efflux transporters, P-glycoproteins (i.e., Pgp; Mdr1) and multidrug resistance-associated proteins (Mrps) in rodents. Pgp and Mrp transporters are members of the ATP-binding cassette (ABC) superfamily, are regulated by AhR, CAR, and PXR, and play important roles in the efflux of xenobiotics and THs into the bile for elimination.

Binding to Thyroid Receptors

Limited evidence in fish and other vertebrates supports that PBDEs may, in part, be mediating effects on the thyroid by altering TR expression and signaling. The transcriptional activity of nuclear TRs is thought to be mediated by both the presence and absence of T3 due to its ability to bind to TRE regions of regulated genes in both the presence and absence of ligand. As in other vertebrates, two genetically distinct receptors TRα and TRβ have been identified in teleost fishes, including zebrafish,[174,175] flounder (*P. olivaceus*)[176]; goldfish,[177] fathead minnows,[178,179] Nile tilapia (*O. niloticus*),[180] and Atlantic salmon.[180] Additional receptor subtypes with the capacity to bind TH have also been identified attributable to gene duplication and alternative mRNA splicing. Two trα genes, *thraa* (original) and *thrab* (duplication) have been described in zebrafish with the *thraa* gene shown to encode two protein variants, TRαA1 and TRαA1–2.[181,182] Two TRβ isoforms have also been identified in teleosts.[175,180] While TR variants arising from TRα and TRβ have also been described in other vertebrates, including humans, the general structure and function of TRs appears well conserved across vertebrates, which has been the topic of several reviews.[183-185]

Questions remain, however, concerning whether and how parent PBDEs and their OH-BDE metabolites interact with and bind to TRs. For instance, BDE-47 was reported to not interact as either an agonist or antagonist with TRβ1 in an in vitro binding assay and did not interfere with TRβ-responsive gene expression.[186] However, altered expression in thyroid-responsive genes was observed in the brain and livers of rodent pups exposed perinatally to BDE-47, suggesting that BDE-47 operated through alternative mechanisms to TRβ signaling. Studies in rat pituitary GH3 cell proliferation assays have shown BDE-127 and BDE-185 to be TR agonists while BDE-206 was a TR antagonist.[167,187,188] Cell based assays have shown that some OH-BDEs, including 3-OH-BDE-47, can inhibit T3 binding to TRs by antagonizing the receptor, whereas several other parent PBDEs and OH-BDEs have shown no TR affinity.[189,190] Another study showed TR antagonistic activity for BDE-209, -153, -154, -100, and PentaBDE.[191] Limited evidence has shown some OH-BDEs to behave as weak TR agonists.[192-194] It has been suggested that hydroxy moieties in the 3 or 4 position of the phenyl ring along with two adjacent bromine substituents are necessary for OH-BDE to bind to TRs.[189] In addition, recent molecular docking assays have shown that OH-BDEs may have varied interactions with TR binding pockets depending on the degree of bromination with lower OH-BDEs (e.g., 6-OH-BDE-47, 5-OH-BDE-47) showing weak TR agonism while higher OH-BDEs (e.g., 3-OH-BDE-100, 3′-OH-BDE-154) antagonized TRs.[194] Taken together, data on TR binding of PBDEs and OH-BDEs continue to be inconsistent and have demonstrated both antagonistic and weak agonistic activities toward TRs, as well as no interactions, that may be attributable to hydroxylation and bromination patterns that influence binding geometries with the receptor.

PBDEs also may alter patterns of TR-responsive gene transcription, although this remains understudied. For instance, reductions in relative mRNA transcript abundances of brain transcription element binding protein (BTEB) were measured in adult fathead minnows exposed to BDE-47.[154] The downregulated expression of BTEB, which is a thyroid-responsive transcription factor involved in neurogenesis, was accompanied by declines in circulating T4 and reductions in *trβ* gene transcripts in the brains of treated minnows, suggesting that hypothyroidism in BDE-47 treated fish may elicit downstream effects on neurogenic capacity in adults. Similarly, in primary rat cerebellar granule cell cultures, BDE-99 was found to disrupt *trα1* and *trα2* mRNA transcript abundances, alter TR-responsive gene transcription (e.g., brain-derived neurotrophic factor), and increase the production of reactive oxygen species.[195] Finally, a study with CV-1 cell cultures measured suppressed TR binding with TREs through the DNA binding domain (vs. between THs and TRs) upon exposure to several PBDEs and OH-BDEs, with BDE-209 showing the greatest suppression at the lowest dose.[191] The suppressed TR-TRE binding was then shown to inhibit TH-dependent dendrite arborization of cerebellar Purkinje cells, suggesting TR-TRE mediated impacts on PBDE neurotoxicity.

Recently, a second potential binding site for T3 and T4 was suggested in the ligand binding domain (LBD) of TRα [196]. This second binding site was identified on the surface of the TRα LBD in the same region where the F-domain (i.e., additional C-terminal amino acids) is located in some species. While human TRs do not appear to have F-domains, it has been identified within the TRαA1 isoform of zebrafish where it has been shown to constrain transcriptional activity by altering TR coactivator recruitment.[182] Thus, one hypothesis put forth is that this second binding site within TRα may serve to suppress TR activation when elevated concentrations of TH are present.[196] While more work is needed, this second binding site could also play a role in mediating how environmental contaminants like PBDEs interact with TRs and alter the functioning of these nuclear receptors.

Neurodevelopmental Toxicity

Limited evidence of PBDE effects on neurodevelopment of fishes has been observed in early life stages of fish (zebrafish typically) for a subset of PBDE congeners. Specifically,

Table 5. PBDE neurodevelopmental/developmental malformations measured in teleost fish

Species	Treatment	Route	Dose/Duration	Effects Observed	Ref
Zebrafish (Larv.)	BDE-47	Aqueous	0, 1.25, 5, 20 μM; 6–96 hpf	Impaired motor behavior ↓ touch-response, swimming speed Inhibited axon growth	200
Zebrafish (Larv.)	PentaBDE (DE-71)	Aqueous	0, 31, 68.7, 227.6 μg/l; 2–120 hpf	Altered behavior (light-dark stimulation) ↑AChE activity, ↑Ach; ↓ mRNA transcripts for MBP, a1-tubulin, Shh	197
Zebrafish (Larv.)	PentaBDE (DE-71)	Parental	0.16, 0.8, 4.0 μg/l; 150 d	No effect on F1 hatching success, survival, or malformations; decreased locomotor activity (light-dark stimulation) ↓AChE activity, ↓ mRNA transcripts for MBP, GAP-43, GFAP, α1-tubulin, SYN2a; ↓ protein levels of α1-tubulin, SYN2a	198
Zebrafish (Adu. females)	PentaBDE (DE-71)	Aqueous	0.45 μg/l, 9.6 μg/l; 60 d	↓ retinyl ester protein; ↓ CRBP mRNA transcripts (GI); ↑ retinoids (eyes, ovaries, eggs); ↑ CRBP mRNA transcripts (liver, eyes); ↓ retinal dehydrogenase, ↑ CYP26A (eyes)	220
Zebrafish (Larv.)	BDE-209	Spiked sediment	12.5 mg/kg; 4 – 192 hpf	Impaired motor behavior (light/dark stimulation) In silico profiling: BDE-209 binding to neurologically active cocaine esterase, AChE, 5HTR2A, 5HTR2C, 5HTR3A, and tubulin α1A	6
Zebrafish (Adu., offspring)	BDE-209	Aqueous	0.001 – 1 μM; 150 dph (Adu); bred at 120 dph	Parent: ↑ mortality (~44% high dose); Neg ctrl mortality ~38%; PBDE bioaccumulation (congeners not specified) Offspring: Delayed hatching, motor neuron development, loose muscle fibers, slow locomotion; hyperactivity (light-dark test)	205
Zebrafish (Larv.)	BDE-47	Aqueous	100 – 5000 μg/l; 3–168 hpf	Delayed hatching, reduced growth, dorsal curvature, impaired CSF flow Cardiac toxicity at 96 hpf (tachycardia, arrhythmias)	202
Zebrafish (Larv)	BDE-49	Aqueous	4 – 32 μM; 5 and 24 hpf	Dorsal curvatures, cardiac toxicity (reduced heart rate); neurobehavioral effects (impaired touch-escape responses)	204
Mummichog (Larv., Juv.)	PentaBDE (DE-71)	Aqueous	0.001 – 100 μg/l; 0–7 hpf	Delayed hatching; No major deformities but tail curve asymmetry; ↓ activity; impaired fright response (Larv) Impaired learning (Juv.)	98
Zebrafish (Larv.)	BDE-28, -47, -99, -100, -153, -183	Aqueous	0.635 – 10 mg/l; up to 168 hpf	Swimming rate ↑ (96–120 hpf), ↓ (168 hpf) ↓ swimming rates (BDE-47; 168 hpf) Developmental deformities (dorsal curvature at 120 hpf) w/mortality (BDE-28, -47, -99, -100) No effects w/BDE-153, -183	199

α1-tubulin = neuron microtubulin protein; ACh = acetylcholine; AChE = acetylcholinesterase; BTEB = basic transcription element-binding protein; CRBP = cellular retinal binding protein; CSF = cerebral spinal fluid; dep = depuration; dpf = days post fertilization; dph = days post hatch; dio = iodothyronine deiodinase; GAP-43 = growth associated protein 43; GFAP = glial fibrillary acidic protein; HDT = highest dose tested; hpf = hours post fertilization; MBP = myelin basic protein; Shh = Sonic hedgehog; SYN2a = synapsin IIa

neurodevelopmental abnormalities, including impaired normal motor behavior and inhibited neuron growth and differentiation, as well as morphological deformities have been measured in zebrafish larvae exposed to: PentaBDE[157,197,198]; a mixture of BDE-47, -99, -100, -153, and -183[199]; BDE-47,[200-203] BDE-49[204]; and BDE-209[205] (Table 5).

PentaBDE Neurotoxicity

For instance, in 96-hpf zebrafish parentally exposed to the PentaBDE mixture, several genes involved in central nervous system development were downregulated, including α1-tubulin, synapsin IIa, and myelin basic protein.[198] This decline in mRNA expression was accompanied by reduced protein expression of α1-tubulin and synapsin IIa as well as reduced locomotor activity among treated larvae. In another study by this same research group, the PentaBDE commercial mixture also caused a downregulation in mRNA transcript abundances of sonic hedgehog (Shh).[197] This suggests a possible contributory role for thyroid dysregulation in some PBDE related neurodevelopmental toxicity as the Shh pathway, as well as its coreceptors patched (Ptc) and smoothened (Smo), have been shown to be regulated by THs in embryonic forebrain signaling and development in mammals.[206] Only one study has examined neurotoxicity endpoints in a fish species beyond zebrafish. This study, conducted in mummichogs (*F. heteroclitus*) detected hindered behavior and learning ability, as well as dorsal curvatures, in fish exposed to PentaBDE.[98]

BDE-47, BDE-49, and BDE-209 Neurotoxicity

In BDE-47 exposed zebrafish, delayed hatching, neural defects, and cardiac arrhythmias were measured at 168 hpf in larvae exposed to 5 mg/l of BDE-47.[202] The cardiac toxicity and dorsal curvature deformities observed in this study were coupled with reduced flow rates of cerebrospinal fluid in neural tubes and brain ventricles of the hindbrain. The reduced flow rates of cerebrospinal fluid were postulated to be related to the dorsal curvatures that then possibly contributed to the measured neurotoxicity. Dorsal curvatures, attenuated heart rates, and impaired touch-escape responses were also measured in zebrafish larvae exposed to another tetra PBDE congener BDE-49.[204] Moreover, neurodevelopmental impairments, including delayed hatching and hindered motor neuron development, loose muscle fiber deformities and slow locomotion, and hyperactivity under a light/dark stimulation test, have also been observed in zebrafish larvae exposed maternally to BDE-209.[205] These behavioral findings with BDE-209 are consistent with another recent study in which zebrafish larvae exposed to sediment spiked with 12.5 mg/kg of BDE-209 from 4–192 hpf experienced hyperactive responses to light stimulation that may be linked to impaired neurodevelopment.[6] Additional in silico binding assays in this study also predicted BDE-209 binding with several human proteins involved in neurological functioning, including: tubulin α1A involved in microtubule formation; acetylcholinesterase (AChE) involved in the breakdown of acetylcholine; 5HTR2A, 5HTR2C, and 5HTR3A, which are serotonin receptor system genes; and cocaine esterases.

Toxicity Mechanisms and Thyroid Interactions

In mammals, like in fish, neurodevelopmental toxicity of PBDEs is also an important toxicological endpoint of concern. For instance, PBDEs (BDE-47, -99, and -100) measured in umbilical cord blood of women have been found to be correlated with reduced performance of gestationally-exposed children (aged 0–6) on mental performance tests.[207] Moreover, maternal prenatal and childhood PBDE exposures have been associated with reduced attention, fine motor coordination, and cognition (declines in IQ scores) among a California cohort of Mexican-American children.[64] A substantial number of studies in rodents, spanning different laboratories, have demonstrated also that PBDEs can elicit adverse neurobehavioral outcomes in early development.[208-210] Recent in vitro work has even shown that the prominent OH-BDE metabolite in humans, 6-OH-BDE-47, can disrupt adult neurogenesis by inhibiting neuronal differentiation and oligodendrocyte differentiation, proliferation, and survival of primary cultured adult neural stem/progenitor cells isolated from the brains of adult mice.[213] Other in vitro study has shown that the hydroxylated metabolites of BDE-47 may disturb intracellular calcium release.[214]

Some neurological deficits and alterations measured in fish[154] and rodents[191,211,212] have been accompanied by reductions in circulating T4 and altered TH signaling, suggesting that one of the contributing neurotoxicity mechanisms may proceed through PBDE interference with TH regulation and signaling. However, PBDE neurotoxicity has been observed absent impacts on TH regulation, suggesting other mechanistic pathways. Indeed, a growing body of evidence suggests that PBDE mechanisms of neurotoxicity may operate by several pathways that include disrupted TH signaling, altered cholinergic neurotransmissions[215,216]; impaired neuronal proliferation and plasticity,[191,214,217] and oxidative stress.[218,219] While the underlying mechanisms of PBDE neurotoxicity are unclear, continued testing in fish would be informative to better understand these underlying mechanisms of PBDEs effects on the development and functioning of the central and peripheral nervous systems of vertebrates.

Reproductive Toxicity

PBDE impacts on fish reproduction and reproductive development have been evaluated in a limited number of studies and congeners (**Table 6**). Studies that have examined PBDE effects on fish reproduction have presented mixed evidence of reduced fecundity, spawning, hatching success, and offspring survival as well as impaired fertility, particularly among male fish, with PBDE-induced alterations in spermatogenesis, declines in sperm counts, and feminization possibly playing important roles.

PentaBDE Reproductive Effects

Reduced fecundity and larval survival have been measured in zebrafish exposed orally to the PentaBDE mixture purified of PBDDs/PBDFs.[96] Reductions in larval survival in this and other studies may be partly attributable to maternal transfer of PBDEs to eggs, which has been shown in zebrafish and marine medaka (*O. melastigma*) and could hinder normal developmental progression.[221,222] A study in zebrafish adults with the PentaBDE mixture reported sex-specific alterations in the relative expression of genes encoding an array of reproductive hormones and receptors along the HPG axis, as well as disruption in circulating levels of E2, T, and 11-keto-testosterone in males.[223] In a second related study, this same research group also measured PentaBDE-induced reductions in spawning, fertilization, and hatching success along with reduced larval survival and higher percentages of male offspring.[224] These reproductive impairments were accompanied by altered counts of spermatogonia, spermatocytes, and spermatids in the testis of treated fish. In some contradiction, however, this study reported an increase in the gonado-somatic index (GSI) in treated males.

BDE-47 Reproductive Effects

With regard to constituents of the PentaBDE commercial mixture, most of the limited research to date that has studied PBDE impacts on reproduction has been conducted with BDE-47. Reduced spawning has been observed in adult fathead

Table 6. PBDE reproductive alterations measured in teleost fish

Species	Treatment	Route	Dose/Duration	Effects Observed	Ref
Atlantic salmon (*S. salar*; Juv.)	PentaBDE, OctaBDE mixtures	Diet	10 mg/kg bw (d 1), 50 mg/ kg bw (d 4); 7 d, 14 d	No effects on protein expression/activity of Vtg, zona radiata, CYP1A	75
Zebrafish (Juv.)	BDE-47	Diet	100 ng/g; 0.5–1 mg food/fish-d; 20–60 dph	No significant change in Vtg mRNA	95
Zebrafish (Adu.)	PentaBDE (DE-71)	Aqueous	2, 300, 500 ng/l (measured); 120 dpf	Gene Expression: Males: ↑ GnRH, ERβ (brain); ↑ FSHβ, LHβ (pituitary); ↑ FSH-R, LH-R, CYP19a and ↓ CYP11a, 3β-HSD (testis); ↓ ERα, AR, Vtg (liver) / Females: ↑ GnRH and ↓ ERβ (brain); ↑ FSHβ, LHβ and ↓ GnRH-R (pituitary); ↑ 3β-HSD (ovary); ↑ ERα, AR (liver) / Sex hormones: ↓ E2 (males (high dose only) and females); ↑ T, 11-KT; ↑ T/E2 and 11-KT/E2 (males)	223
Zebrafish (Adu.)	PentaBDE mixture (DE-71)	Aqueous	2, 300, 500 ng/l (measured); 120 dpf	Reduced spawning, fertilization, hatching, larval survival; ↑ GSI Increased malformations and percentages of male offspring	224
Zebrafish (Adu., offspring)	BDE-209	Aqueous	0.001 – 1 µM; 150 dph (Adu); bred at 120 dph	↓ male/female GSI; ¯sperm count, motility Offspring: Delayed hatching, motor neuron development, loose muscle fibers, slow locomotion; hyperactivity (light-dark test)	205
European flounder (*P. flesus*; Adu.); Zebrafish (Juv.)	PentaBDE (DE-71 purified of PBDD/Fs)	Spiked sediments, Diet	0+0.014 -700+14000 µg/g TOC + µg/g lipid; 101 d (flounder) 0–500 µg/l; 30 d (zebrafish)	↓ ovarian aromatase (CYP19) activity (flounder); ↓ fecundity (zebrafish)	96
Fathead minnow (Adu. Breeding pairs)	BDE-47	Diet	2.4 µg/pair/ day; 12.3 µg/pair/ day; 21 d	↓ mature spermatozoa	154
Chinese rare minnow (*G. rarus*; Adu., Larv.)	BDE-209	Aqueous	0 – 10 µg/l; 21 d	↓ spermatogenesis	155
Fathead minnow (Adu. Breeding pairs)	BDE-47	Diet	28.7 ± 1.6 µg/pair (bioencapsulated artemia); 25 d	Spawning ceased by 2-wks of exposure Reduced fecundity > 50% reduction in sperm counts No change in GSI, LSI; reduced condition, males	108
Fathead minnow (Adu. males)	BDE-209	Diet	95 ng/g ww food and 10 µg/g ww food at 3% bw/day; 28 d with 14 d dep	↓ GSI at both low and high dose that extended into 14-d dep PTU positive control did not affect GSI	62
Zebrafish (embryos)	BDE-28, BDE-183, BDE-209	Diet, Maternal	10 and 100 nmol/g food at 2% bw/day; 42 d	All three PBDEs transferred to eggs (BDE-28 > BDE-183 > BDE-209) Egg/maternal fish concentration ratios significant > 1.0 for BDE-183, BDE-209	221
Rainbow trout (Adu. Males)	BDE-47	Diet	55 µg/kg-day; 17 d	No effects on embryonic survival at first cleavage (0.5 dph) or during eye development (19 dph)	225
Atlantic salmon (Juv. males)	BDE-47, -153, -154 (alone and mixture)	Hepatocytes	0.01 – 100 µM; 48 h	Disturbed glucose homeostasis (PBDE mix, BDE-153); Altered cell proliferation processes (PBDE mix) Estrogenic responses (↑ Vtg; ZP3 mRNA) in males (BDE-47, PBDE mix)	226
Marine medaka (*O. melastigma*; embryos)	BDE-47	Diet, Maternal	Breeding pairs: 1.3 ± 0.2 µg/day; 18 d	Maternal transfer of BDE-47 ↑ BDE-47 to 25 ng/egg (day 18) Maternal concentrations BDE-47 < males	222
Zebrafish (Adu., offspring)	PentaBDE (DE-71)	Parental, Aqueous	1, 3, 10 µg/l (parents and offspring) + (parent alone); 5 min to sexual maturation (parents), 5 and 10 dph (offspring)	↓ hatching rate ↑ malformation rates to offspring exposed parentally; DE-71 exposures by parental transfer plus directly to offspring worsened malformations	158

11-KT = 11-keto-testosterone; 3β-HSD = 3-β-hydroxy steroid dehydrogenase; CRH = corticotrophin releasing hormone; CSF = cerebral spinal fluid; dep = depuration; dpf = days post fertilization; dph = days post hatch; E2 = estradiol; ER = estrogen receptor; FSH = follicle stimulating hormone; FSH-R = follicle stimulating hormone receptor; GSI = gonado-somatic index; GnRH = gonadotropin releasing hormone; HDT = highest dose tested; hpf = hours post fertilization; LH = luteinizing hormone; LH-R = luteinizing hormone receptor; PTU = 6-propyl-2-thiouricil; t = testosterone; TOC = total organic carbon; Vtg = vitellogenin; ZP3 = zona pellucida 3

minnow male/female pairs exposed orally to ~14 µg/fish of BDE-47 for 25 d, with reproduction completely ceased within 10 d of exposure.[108] The impaired reproduction may have been attributable to selective toxicity in male fathead minnows as decreased mature spermatozoa and reduced condition factors were noted in these fish. In another study, declines in mature spermatozoa also were measured, but with no effect on fecundity, in adult male fathead minnows exposed orally to ~6 µg/fish-day of BDE-47 for 21 d.[154] BDE-47 also elicited no effects on the morphological development of offspring from adult male rainbow trout exposed to 55 µg/kg-day of BDE-47 for 17 d and then bred with untreated females.[225] These data suggest that other components in the PentaBDE mixture in addition to BDE-47 may play an important role in affecting fecundity, embryonic development, and adult male reproduction.

PBDE Effects on Male Fish Reproduction

Some studies have suggested a role for PBDEs in the feminization of male fish. Specifically, dose-dependent increases in relative mRNA transcripts encoding vitellogenin (Vtg; egg yolk precursor protein) and zona radiata protein (eggshell protein) were measured in hepatocytes of juvenile male Atlantic salmon (*S. solar*) exposed to BDE-47 and a PBDE mixture (BDE-47, 153, -154).[226] However, some of these in vitro findings have not been reproduced with in vivo studies of juvenile Atlantic salmon exposed to the PentaBDE mixture[75] or in zebrafish larvae exposed to BDE-47,[95] whereby no change in the expression of Vtg mRNA transcript abundances were detected. Thus, while the in vitro evidence is limited because it may not account for the extensive and coordinated interactions among cells and tissues, conversely the in vivo research conducted to date targeting PBDE effects on male fish reproduction has only been in two species, targeting largely transcriptional changes in immature animals for just a limited number of genes. In addition, very little is known about the potential reproductive effects of BDE-209 in fishes but limited evidence suggests alterations to some reproductive endpoints in male fish. Specifically declines in spermatogenesis were observed in male Chinese rare minnows (*G. rarus*) exposed aqueously to BDE-209,[155] and reductions in the gonadal somatic index (GSI) have been measured in adult male fathead minnows exposed to BDE-209[62,205] with evidence of declining sperm counts in some of this research.[205] Taken together, more research is necessary to understand whether PBDEs play a role in eliciting potential feminization and reproductive impairments in male animals.

Toxicity Mechanisms and Thyroid Interactions

Fish have evolved diverse reproductive strategies that are closely integrated with gonadal differentiation and functioning and demonstrate sensitivity to external environmental cues, such as photoperiod and temperature. While there are important differences between reproduction in mammalian and non-mammalian vertebrates, reproduction in jawed vertebrates is controlled by the HPG axis and the structure of this endocrine system is highly conserved. It is possible that there could be shared pathways of PBDE impacts on mammalian and non-mammalian vertebrate sex steroid synthesis and gonadal development and functioning. It is also possible that there may be important species- and sex-specific differences in reproductive responses to PBDEs as current studies are limited by teleost species and focused largely on altered reproduction in males with less known about reproductive effects in female fish.

For instance, in female fish, declines in ovarian aromatase (CYP19) activity were measured in European flounder (*P. flesus*) exposed to the PentaBDE mixture purified of PBDDs/PBDFs, suggesting that PBDEs may be altering pathways of steroidogenesis in female fish.[96] Somewhat consistent with these findings, in vitro testing also has shown potential inhibitory effects of OH-PBDEs on CYP19 and CYP17 in human placental microsomes[227] and human adrenocortical carcinoma cells.[228,229] Several OH-BDEs have also been found to bind with the estrogen receptor (ER) with the general trend that *para*-OH metabolites displayed the highest affinity for ERs with the lower OH-BDEs (1–4 bromines) tending to act as weak agonists while higher OH-BDEs (4 bromines or more) had antagonistic properties.[189,230,231] In addition, rodent studies[232-236] and cell-based assays[187,237] have shown that some PBDEs (PentaBDE, BDE-47, -99, -100, and -209) may be estrogenic and/or induce feminization in male animals by anti-androgenic pathways. In humans, epidemiology studies have measured PBDE associations with: cryptorchidism[238]; early onset of menarche[239]; decreased testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) in adult men[151]; increased E2 in 3-mo old boys (BDE-154)[240]; and decreased sperm counts and testis size in young adults (BDE-153).[241] Taken together, there appear to be important common mechanistic pathways of PBDE effects on vertebrate reproduction that are not well understood at this time.

While some evidence points to PBDEs affecting the reproductive health of fish and other vertebrates, few studies have examined interactions between thyroid and reproductive functioning. Thus, questions remain as to whether PBDE effects on reproduction are being mediated directly and/or indirectly by altered TH regulation. In mammals, both hypothyroidism and hyperthyroidism have been shown to impair reproductive physiology and lower fertility.[242] An early review described important interactions between TH regulation and reproductive physiology in fishes.[243] More recently, studies in goldfish (*C. auratus*) and zebrafish suggest that THs may have important inhibitory effects on teleost reproductive functioning at different levels of the HPG axis, including by: inhibiting pituitary LH and FSH; and reducing steroidogenesis and gonadal aromatase expression.[244,245] Recent evidence, in some contrast, also supports a stimulatory role for THs in the proliferation of sertolli cells and spermatogonia in zebrafish testes.[246]

Little is known about the role of PBDE-induced TH disruption in potential reproductive toxicity in fishes or other vertebrates. One hypothesis is that PBDEs could be mimicking THs that is in turn leading to altered steroidogenesis and steroidal

hormone regulation among exposed animals, although this has not been examined. However, in data collected in male fathead minnows exposed orally to BDE-209, the model goitrogen 6-propyl-2-thiouracil (PTU), which was used as a positive control, reduced TH levels as predicted but had no effect on the GSI, unlike BDE-209 which caused substantial reductions in the GSI. These results suggest that BDE-209 reproductive effects in male fish could be acting through thyroid-independent pathways.[62] Still though, it remains unclear from the limited data whether PBDE effects on reproduction are mediated by directly impairing HPG functioning or whether these effects are also mediated in cross-talk with perturbations of the thyroid.

Research Needs and Conclusions

A substantial body of evidence suggests that PBDE metabolism in teleost fishes proceeds through reductive debromination pathways. Studies report both the presence and absence of OH-BDE metabolites forming in fish, and given their bioactivity, it would be informative to investigate further whether these metabolites form in vivo in fish. It might be expected that the OH-BDEs, if they are being produced by fish, would be found at higher concentrations in the blood and highly perfused tissues like the liver where they are formed mostly. Related to this, questions remain as to the identity and kinetics of the enzymatic biotransformation pathways of PBDE metabolism in fish with some indirect evidence implicating Dio enzymes. In mammals, PBDEs appear to operate predominantly through AhR-independent toxicity pathways. However, the extent to which these pathways (e.g., CAR, PXR) are operational in fish exposed to PBDEs is less clear, but potentially relevant to the hypothyroidism observed given their additional role in TH metabolism and elimination.

PBDEs have been shown now to disrupt TH regulation and signaling in several teleost species with mechanisms of action that proceed through multiple pathways depending on the congener, fish life stage, and tissue type, including by: enhancing the metabolism and elimination of THs; binding competitively with plasma and membrane bound transporter proteins; altering interactions of T3 ligand with nuclear TRs; disrupting Dio activity; and altering the transcription of genes involved in TH production, transport, and genomic signaling. Despite expanding knowledge of these thyroid disruption mechanisms, there continues to be a limited understanding of PBDE impacts on thyroid-response gene expression and downstream apical endpoints of concern, including neurodevelopment and reproduction. PBDE effects on neurological, developmental, and reproductive physiology have been indicated across fish and other vertebrates exposed to PBDEs, suggestive of common toxicity pathways that remain unclear. Related to this, additional work is needed to understand whether there exist localized compensatory or adaptive physiological responses of the thyroid system to TH insufficiency caused by PBDE exposures. Continued study of these apical endpoints of concern in fish would be helpful for not only understanding potential toxicities in free ranging fishes, but also would contribute to understanding PBDE mechanisms of toxicity in humans.

The thyroid endocrine systems of fish and mammals are well conserved and similar in structure and function, supporting the relevance of fish as models for understanding PBDE effects more broadly across vertebrate taxa. However, there are differences between mammalian and non-mammalian thyroid signaling that could have implications for using fish as models for evaluating PBDE thyroid toxicity in higher level animals. One important difference is that unlike in mammals, the fish thyroid may not to be centrally directed through the HPT but rather appears to rely strongly on localized peripheral tissues for T3 production and regulation. There is evidence for this dominant peripheral signaling in mammalian models as well, but nonetheless demonstrates the need to continually evaluate the choice of animal model when studying chemically induced thyroid disruption. For some PBDE congeners, evidence in fish demonstrates thyroid dysregulation at low doses (i.e., ~ppb levels) that roughly compares to levels detected in the environment and tends to be lower than doses typically administered in rodent studies. Additional low dose studies are needed to determine whether non-monotonic dose responses are occurring in fish and other vertebrates exposed to PBDEs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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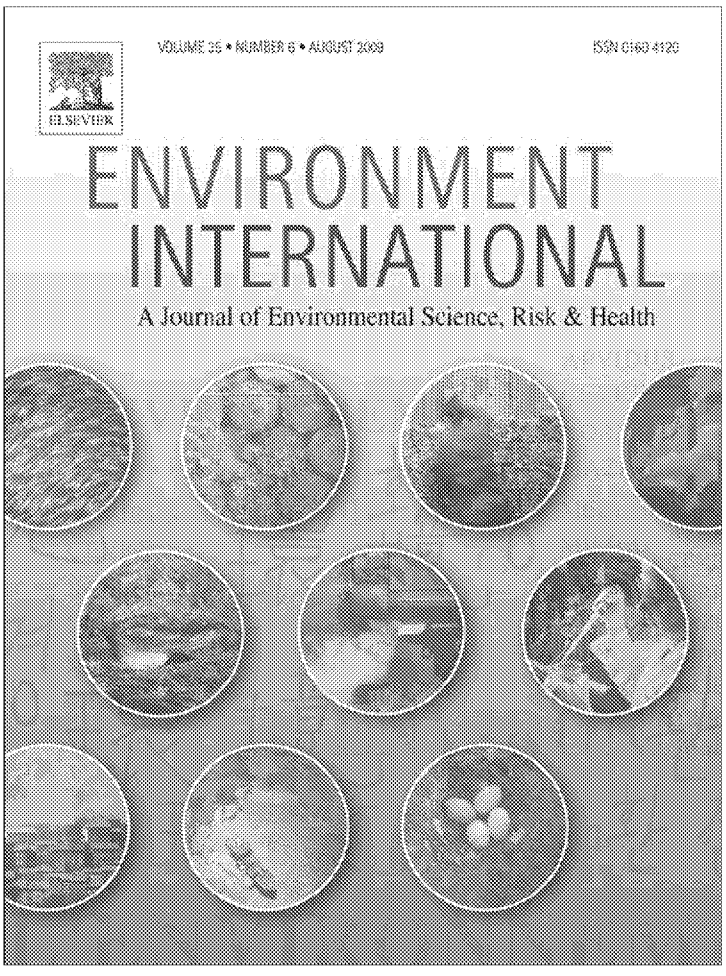
Peer Review Publication 5:

Noyes PD, McElwee MK, Miller HD, Clark BW, Van Tiem LA, Walcott KC, Erwin KN, Levin ED. 2009. The toxicology of climate change: Environmental contaminants in a warming world. *Environment International* 35(6):971-986.

Basis for inclusion and scientific impact:

Issues surrounding the human health and ecological effects of chemical exposures coupled with climate change have not been well characterized but are becoming an increasingly important issue. The Intergovernmental Panel on Climate Change (IPCC) in its most recent 5th Assessment Report (https://www.ipcc.ch/report/ar5/) published in 2014 describes that climate change coupled with air pollution (and other risk factors, such as heat stress, precipitation extremes, and drought) are projected to increase risks for people, assets, and economies, particularly for those urban populations lacking essential infrastructure and services. Similarly, the IPCC reports that terrestrial, freshwater, and marine species face increased extinction risk due to climate change and its interactions with pollution and other factors. In light of these types of growing concerns about climate-toxicology interactions, I spearheaded a collaborative project to complete and publish an in-depth review of the current evidence describing the effects of climate change on the distribution and toxicity of chemicals. At the time this manuscript was published in 2009, there had been very little attention focused on this topic. This paper was a collaborative effort among several scientists at Duke University. I was the lead author on the sections describing climate change effects on the environmental fate and distribution of chemical as well as the review of the interactive effects of climate change and pollution on human health. I was also the lead writer describing the effects of climate change on chemical toxicokinetics and toxicity pathways to wildlife. I managed the peer review process including editing the manuscript and responding to comments. The results of this work have been gratifying because it has played an important role in drawing attention to the influence of climate change on chemical exposure and toxicity, and conversely, the influence of chemical exposures on exacerbating climate risk. It contributed to informing the basis for a global SETAC Pellston workshop to assess and develop approaches to consider climate change in chemical risk assessment, which I had the pleasure of participating (See Section D). As further evidence of its impact, Google Scholar reports that it has been cited over 390 times in peer review publications.



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Review article

The toxicology of climate change: Environmental contaminants in a warming world

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ABSTRACT

Climate change induced by anthropogenic warming of the earth's atmosphere is a daunting problem. This review examines one of the consequences of climate change that has only recently attracted attention: namely, the effects of climate change on the environmental distribution and toxicity of chemical pollutants. A review was undertaken of the scientific literature (original research articles, reviews, government and intergovernmental reports) focusing on the interactions of toxicants with the environmental parameters, temperature, precipitation, and salinity, as altered by climate change. Three broad classes of chemical toxicants of global significance were the focus: air pollutants, persistent organic pollutants (POPs), including some organochlorine pesticides, and other classes of pesticides. Generally, increases in temperature will enhance the toxicity of contaminants and increase concentrations of tropospheric ozone regionally, but will also likely increase rates of chemical degradation. While further research is needed, climate change coupled with air pollutant exposures may have potentially serious adverse consequences for human health in urban and polluted regions. Climate change producing alterations in: food webs, lipid dynamics, ice and snow melt, and organic carbon cycling could result in increased POP levels in water, soil, and biota. There is also compelling evidence that increasing temperatures could be deleterious to pollutant-exposed wildlife. For example, elevated water temperatures may alter the biotransformation of contaminants to more bioactive metabolites and impair homeostasis. The complex interactions between climate change and pollutants may be particularly problematic for species living at the edge of their physiological tolerance range where acclimation capacity may be limited. In addition to temperature increases, regional precipitation patterns are projected to be altered with climate change. Regions subject to decreases in precipitation may experience enhanced volatilization of POPs and pesticides to the atmosphere. Reduced precipitation will also increase air pollution in urbanized regions resulting in negative health effects, which may be exacerbated by temperature increases. Regions subject to increased precipitation will have lower levels of air pollution, but will likely experience enhanced surface deposition of airborne POPs and increased run-off of pesticides. Moreover, increases in the intensity and frequency of storm events linked to climate change could lead to more severe episodes of chemical contamination of water bodies and surrounding watersheds. Changes in salinity may affect aquatic organisms as an independent stressor as well as by altering the bioavailability and in some instances increasing the toxicity of chemicals. A paramount issue will be to identify species and populations especially vulnerable to climate–pollutant interactions, in the context of the many other physical, chemical, and biological stressors that will be altered with climate change. Moreover, it will be important to predict tipping points that might trigger or accelerate synergistic interactions between climate change and contaminant exposures.

Contents

Abbreviations: CO, Carbon Monoxide; CO_2, Carbon Dioxide; DDT, Dichlorodiphenyltrichloroethane; FMO, Flavin-Containing Monooxygenases; HCB, Hexachlorobenzene; HCH, Hexachlorocyclohexane; HLC, Henry's Law Constant; IPCC, Intergovernmental Panel on Climate Change; NOx, Nitrogen Oxides; OP, Organophosphate; PCP, Pentachlorophenol; PBT, Persistent, Bioaccumulative, and Toxic; PCB, Polychlorinated Biphenyl; PM, Particulate Matter; POP, Persistent Organic Pollutant; SO_2, Sulfur Dioxide; VTG, Vitellogenin; VOC, Volatile Organic Compound.

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1. Introduction

Climate change is an increasingly urgent problem with potentially far-reaching consequences for life on earth. Humans and wildlife are also exposed to an array of chemical, physical, and biological stressors that arise largely from anthropogenic activity, but also from natural sources. One of the consequences of climate change that has recently attracted attention is its potential to alter the environmental distribution and biological effects of chemical toxicants. There is growing awareness of the importance of anticipating the effects of chemical pollution in the rapidly changing environment, and identifying and mitigating effects in those humans and ecosystems most vulnerable.

The U.N. Intergovernmental Panel on Climate Change (IPCC) has completed four assessments covering the evidence, impacts, and mitigation of climate change (IPCC, 2007a,b,c,d,e). They report unequivocal global warming with evidence of increases in global mean air and ocean temperatures, widespread snow and ice melt, and rising global sea level. Temperature is projected to increase 1.8–4.0 °C by the end of the century under a range of probable greenhouse gas emission scenarios with the greatest warming expected at high latitudes. In addition to global warming, some regions, such as North and South America, northern Europe, and northern and central Asia are projected to experience increased precipitation, while others, including southern Africa and Asia and the Mediterranean, are expected to experience substantial droughts. Heat waves, precipitation and storm events are predicted to be more frequent and intense. Oceanic acidification linked to increasing atmospheric carbon dioxide levels is a growing threat to marine organisms and ecosystems.

This article examines how the environmental parameters, temperature, precipitation, and salinity, as altered by climate change, could affect the environmental distribution and biological effects of chemical toxicants. It is intended to provide a broad perspective on the interactions of climate change and chemical behavior/toxicity based on available research, which in some cases continues to be limited. For example, key aspects of climate change and pollutant interactions that merit further study involve describing effects on vulnerable species and populations and revealing the nature of thresholds that might trigger adverse events. While climate change will affect the environmental distribution and toxicity of numerous chemical toxicants, we focus primarily on three major classes of global significance: air pollutants, persistent organic pollutants (POPs), and other pesticides. Air pollution is a global problem, and here we focus on two compounds, tropospheric ozone and particulate matter (PM), as they are potent toxicants of human health concern. POPs are persistent, bioaccumulative, and toxic (PBT) contaminants found ubiquitously in the environment, humans, and wildlife. At present, twelve chlorinated organic chemicals are listed as POPs under the U.N. Stockholm Convention, including several organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT) and toxaphene, as well as the polychlorinated biphenyls (PCBs), dioxins, and furans (UNEP, 2005). Other pesticides, such as atrazine, aldicarb, and chlorpyrifos are of special interest as they are applied in large quantities over a broad area and have a range of toxicological effects. Moreover, pesticide use patterns may change as agriculture and pest species shift in response to climate change.

2. Effects of climate change on contaminant environmental fate and behavior

Climate change will have a powerful effect on the environmental fate and behavior of chemical toxicants by altering physical, chemical, and biological drivers of partitioning between the atmosphere, water, soil/sediment, and biota, including: air-surface exchange, wet/dry deposition, and reaction rates (e.g., photolysis, biodegradation, oxidation in air). Temperature and precipitation, as altered by climate change, are expected to have the largest influence on the partitioning of chemical toxicants. In addition, an array of important processes, such as snow and ice melt, biota lipid dynamics, and organic carbon cycling, will be altered by climate change potentially producing significant increases in fugacity (thermodynamic measure of substance tendency to prefer one phase over another) and contaminant concentrations (MacDonald et al., 2002).

2.1. Altered fate and behavior of air pollutants

It is widely recognized that air quality and climate change are strongly interconnected (IPCC, 2007c,e). Climate change is projected to generally degrade air quality, but for tropospheric ozone and PM, there continues to be uncertainty as to the direction and magnitude of changes in environmental distribution patterns (Aw and Kleeman, 2003; Ebi et al., 2006; IPCC, 2007c,e; Racherla and Adams, 2006).

Tropospheric ozone is generally short-lived and forms in the lower atmosphere from the nitrogen oxide (NOx)-dependent photochemical oxidation of volatile organic compounds (VOCs), carbon monoxide (CO), and sulfur dioxide (SO_2) (Forster et al., 2007). Ozone levels are dictated by emissions of ozone precursors, temperature, water vapor levels, atmospheric circulation patterns, and stratospheric inputs (Denman et al., 2007; Forster et al., 2007; Stevenson et al., 2006). Elevated temperatures generally lead to increased formation of ozone, while increased water vapor generally leads to increased breakdown of ozone. As such, climate change impacts on regional ozone levels will largely be determined by the extent to which temperature, water vapor levels, and air circulation patterns are altered. The interplay of these factors is depicted in Fig. 1, Legend Item A.

While ozone concentrations are projected to increase for many regions, climate change, on a global scale, is expected to generally accelerate tropospheric ozone destruction due to catalyzed photodegradation in the

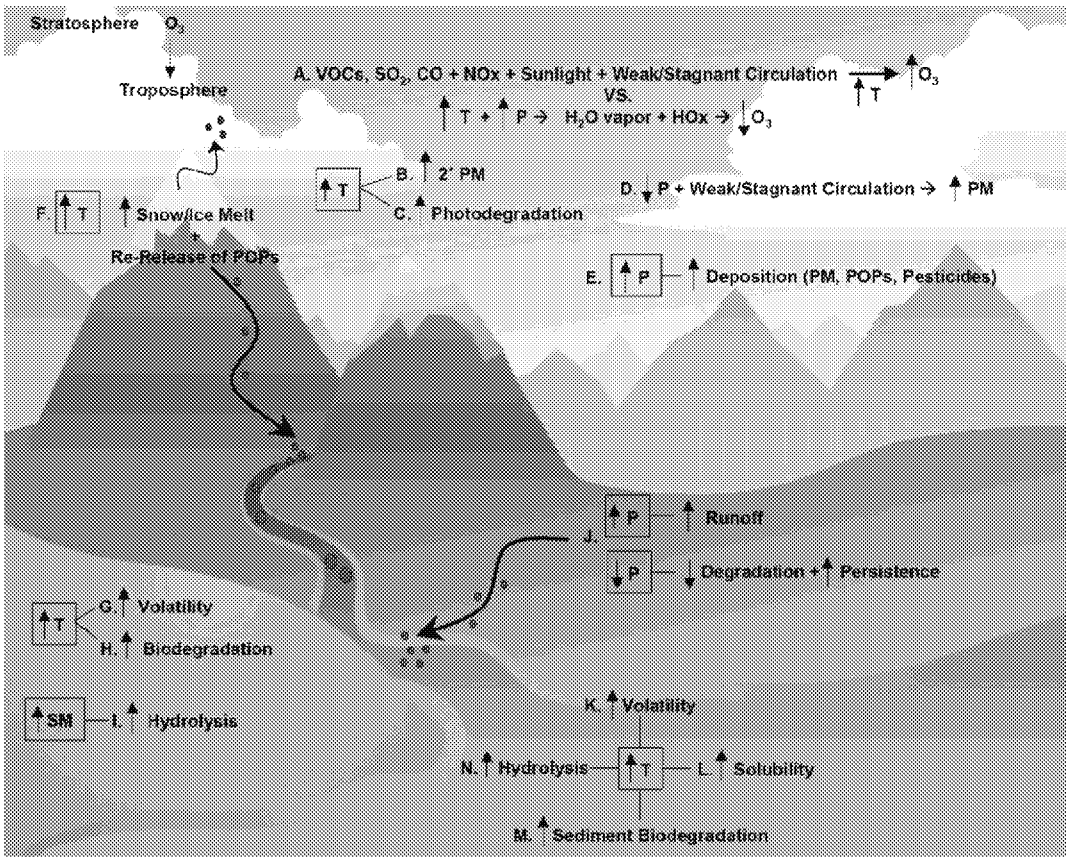

Fig. 1. Effects of climate change effects on the environmental distribution of contaminants. Figure legend: A. Temperature increases coupled with ozone precursors, sunlight, and weak/stagnant circulation will increase the rate of formation of ozone, whereas water vapor will increase ozone destruction. B. Temperature increases may promote the formation of secondary PM. C. Temperature increases may lead to increased photodegradation of POPs. D. Climate change producing increases in precipitation will increase the wet deposition of PM, POPs, and pesticides. E. Declining precipitation coupled with weak/stagnant circulation may increase PM regionally. F. Melting snow, ice, and glaciers will release and remobilize POPs sequestered in these once frozen matrices. G. Temperature increases will enhance the volatility of POPs and other pesticides from soils to the atmosphere. H. Temperature increases will enhance microbial degradation of POPs and pesticides in soils and sediments. I. Enhanced soil moisture will increase hydrolytic degradation of pesticides, but may not affect POPs since they are relatively resistant to hydrolysis. J. Precipitation increases will enhance the potential for pesticide and POP runoff into aquatic system, whereas decreases in precipitation will ameliorate chemical runoff but may increase persistence. K. Water temperature increases will enhance the volatility of POPs and other pesticides from water to the atmosphere. L. Water temperature increases will increase the solubility of POPs making them more apt to be retained in water. M. Water temperature increases will enhance microbial activity increasing the degradation of POPs and pesticides in soils and sediments. N. Water temperature increases will increase the hydrolysis of pesticides to less or more bioactive degradates. 2°PM = Secondary PM; CO = Carbon monoxide; HO*x* = HO_2 + OH; NOx = Nitrogen oxides; O_3 = Ozone; P = Precipitation; PM = Particulate Matter; POP = Persistent Organic Pollutant; SM = Soil Moisture; T = Temperature; VOCs = Volatile organic compounds.

presence of increased atmospheric water vapor. For example, Racherla and Adams (2006) project a 5% decline in *global* tropospheric ozone concentrations in the 2050s from 1990s levels using present day pollutant emission scenarios. Dentener et al. (2006) and Stevenson et al. (2006) estimated future ozone concentrations for 2030 based on current levels of emissions. They calculated that climate change could reduce *global* ozone by 0.5–1.0 ppb over the continents and 1–2 ppb over the oceans.

However, despite estimates of net global declines, several studies project regional scale increases in ozone pollution linked to climate change (Aw and Kleeman, 2003; Cheng et al., 2007; Hogrefe et al., 2004; Langner et al., 2005; Stevenson et al., 2006). For example, tropospheric ozone concentrations are predicted to increase in Southern California as a result of accelerated gas phase reaction rates associated with rising temperatures (Aw and Kleeman, 2003). Similarly, using the IPCC A2 high CO_2 emission scenario, Hogrefe et al. (2004) estimate increases in summertime average daily maximum 8-hour ozone concentrations over the eastern U.S. of 2.7 ppb by the 2020s, 4.2 ppb by the 2050s, and 5.0 ppb by the 2080s.

Models of the New York metropolitan area have been used to estimate average summertime ozone increases from 0.3 ppb in the 1990s to 4.3 ppb by the 2050s (Knowlton et al., 2004). Cheng et al. (2007) modeled future concentrations of various air pollutants (ozone, NOx, SO_2, and suspended particulates) in four south-central Canadian cities (Montreal, Ottawa, Toronto, Windsor) using end of 20th century emission scenarios. They found that a warming climate would increase the number of days in the high ozone category (concentrations ≥81 ppb) by 40–100% by the 2050s and 70–200% by the 2080s, from the current average of eight days. In Europe, increases in tropospheric ozone are projected over central and southern regions predicted to experience precipitation declines (Langner et al., 2005). In contrast, ozone decreases are projected to occur over northern Europe due to increased precipitation.

Climate change-induced shifts in precipitation patterns will also affect PM fate and behavior (Aw and Kleeman, 2003; Forster et al., 2007; Racherla and Adams, 2006). PM consists of both natural and anthropogenic sources of soils, dusts, acids, organic chemicals, and metals. It enters the atmosphere through direct emissions or is formed as secondary particles through atmospheric chemical reactions (Forster et al., 2007). Much of the research on PM fate and behavior focuses on PM_{10} (particles with an aerodynamic diameter ≤10 μm) and more recently on $PM_{2.5}$ (fine particles with an aerodynamic diameter of ≤2.5 μm) as these particle sizes are inhalable and have been shown to be potent toxicants (Forster et al., 2007; USEPA, 2004).

Decreased concentrations of atmospheric fine PM are projected in regions that experience increases in precipitation due to enhanced scavenging of PM by water molecules. Racherla and Adams (2006) estimate that increases in precipitation and wet deposition loss rates could decrease the global burdens and atmospheric residence times of $PM_{2.5}$ by 2–18% by the 2050s (Fig. 1, Legend Item E). However, changes in

other climate variables may also affect PM concentrations. Global warming could increase the formation of secondary PM by catalyzing *in situ* gas phase reactions (Fig. 1, Legend Item B). Aw and Kleeman (2003) modeling of PM interactions with climate change in the Southern California region indicate that non-volatile secondary PM may increase with rising temperatures, but that semi-volatile secondary PM could increase or decrease.

PM concentrations are highly affected by regional emissions, and atmospheric transport of these pollutants can be driven by synoptic-scale (i.e., low or high pressure systems of the lower atmosphere that range on the order of 1000 to 2500 km) weather patterns. For example, Buchanan et al. (2002) investigated the influence of regional weather patterns on PM_{10} concentrations in Edinburgh, Scotland and demonstrated that PM can move well beyond its point source due to these large area dynamics. As climate change is predicted to affect synoptic-scale weather patterns, regional distribution of air pollutants may be affected. In contrast, increases in the frequency of stagnant air events in polluted urban, rural, or industrial settings could enhance the intensity of air pollution (Fig. 1, Legend Item D) (Denman et al., 2007).

2.2. Altered fate and behavior of POPs

Climate change will influence the environmental fate and behavior of POPs by altering the fundamental mechanisms of solvent switching and solvent depletion, and by enhancing contaminant degradation (Brubaker and Hites, 1998; Ma et al., 2004; MacDonald et al., 2002; Meyer and Wania, 2008; Sinkkonen and Paasivirta, 2000; Sweetman et al., 2005; Wania, 1999). Solvent switching involves contaminants partitioning into different chemical phases (solid, liquid, gas) in the direction of thermodynamic equilibrium. While this process can increase concentrations of a contaminant in an environmental compartment (water, sediment, biota, etc.), it cannot produce contaminant concentrations that exceed the thermodynamic equilibrium (MacDonald et al., 2002; Wania, 1999). The effects of global warming on solvent switching can be predicted by considering temperature-driven changes in partitioning constants of POPs, such as Henry's Law Constants (HLC).

In contrast to solvent switching, solvent depletion is a complex process that requires energy and increases fugacity and often contaminant concentrations as solvent concentrations continually decline. Thus, contaminant concentrations in a given environmental compartment can exceed the thermodynamic equilibrium (MacDonald et al., 2002). Examples of solvent depletion processes that may be influenced by climate change include contaminant biomagnification, trophic structure alterations, hydrological processes, and organic carbon cycling. Many transport processes and spatial and temporal variables can influence solvent depletion processes making them difficult to predict (Macdonald et al., 2003; Wania, 1999).

Enhanced volatility and partitioning of POPs to the atmosphere by solvent switching is likely with global warming, as are increases in the rate of contaminant degradation. The warming climate may produce a minor reduction in POP exposure to aquatic biota because of enhanced partitioning from water to the atmosphere as contaminant HLCs rise with increasing water temperatures (Ma et al., 2004; Macdonald et al., 2003). Supporting this hypothesis, elevated air temperatures from 1990–2000 linked to fluctuations of the North Atlantic Oscillation, El Niño-Southern Oscillation, and Pacific North American patterns increased the volatility and atmospheric concentrations of the POPs, hexachlorobenzene (HCB), and PCBs, in the Great Lakes Region, USA (Ma et al., 2004).

Observed temperature increases due to climate change are most pronounced at higher latitudes. The IPCC reports that average arctic temperatures have increased at nearly twice the global average rate in the past 100 years (IPCC, 2007e). POPs are unique in that they can move thousands of miles from their point of release and are often observed at higher latitudes. This observation is explained by the concept of global fractionation (Braune et al., 2005; Breivik et al., 2004; Wania and Mackay, 1996). Most POPs are semi-volatile enough to evaporate at temperate or tropical latitudes, existing as gases or adsorbed to aerosols in the atmosphere. Global atmospheric circulation transports these air masses, containing POPs, to higher latitudes in short jumps coinciding with the seasons. As temperature gradients between high and low latitudes become less pronounced, the temperature-induced global fractionation of POPs to high latitudes could decline (Beyer et al., 2003).

In addition, a decline in atmospheric partitioning and transport of POPs to the poles may result from temperature- and precipitation-accelerated increases in degradation, particularly in the atmosphere and soil (Dalla Valle et al., 2007; Macdonald et al., 2005; Sinkkonen and Paasivirta, 2000; Sweetman et al., 2005; Wania and Mackay, 1996). Dalla Valle et al. (2007) predict that increasing temperatures in Venice Lagoon, Italy will accelerate the degradation of PCB 118 and PCB 180 congeners, 2,3,7,8-tetrachlorodibenzofuran, and 1,2,3,4,7,8-hexachlorodibenzofuran in most environmental compartments. However, these authors note that while elevated temperatures are expected to decrease the fugacity capacity (i.e., indicator of compartment capacity to store a chemical) of most environmental compartments, the fugacity capacity of the air compartment is projected to decline only negligibly. Thus, enhanced atmospheric mobility and long-range transport is predicted in this study. Global warming, however, may also accelerate atmospheric photodegradation of POPs, counter-balancing this atmospheric partitioning (Brubaker and Hites, 1998; Sinkkonen and Paasivirta, 2000).

While atmospheric partitioning of POPs and enhanced degradation are generally predicted with climate change, regional patterns of increased precipitation and ice/snow melt are expected to enhance wet deposition of POPs to aquatic and terrestrial ecosystems (Macdonald et al., 2003; Meyer and Wania, 2008; Wania and Mackay, 1996). Macdonald et al. (2003) note that increases in precipitation will be an important variable driving the distribution of some POPs, such as hexachlorocyclohexane (HCH) and toxaphene, to aquatic systems. Both HCH and toxaphene have HLCs that favor water partitioning. Moreover, snow and snowmelt are powerful drivers in solvent switching and solvent depletion processes that may increase contaminant levels (Macdonald et al., 2003; Meyer and Wania, 2008). Falling snow provides a solvent switching condition under which contaminants can be readily adsorbed to snow surfaces and transported to the ground. As the climate warms and snow melts or sinters, the loss of surface area results in a solvent depleting condition that increases the concentration of contaminant in meltwater. Macdonald et al. (2003) estimate that this process might result in a loss of 10^5 to 10^6 m^2 of surface area for every 1000 kg of snow, which may lead to a substantial increase of POPs in meltwater.

Melting sea ice coupled with expanded open water may also accelerate the rate of exchange of some POPs from air to water. Macdonald et al. (2005) provide a summed PCB congener budget of gas exchange into the Arctic Ocean of 20 metric tons/year, and estimate that reduced Arctic sea ice cover of 50% could result in a proportionate doubling of PCB air to sea exchange. Glaciers have also acted as long-term sinks for POPs and melting of this ice is expected to remobilize these archived pollutants (Fig. 1, Legend Item F) (Blais et al., 2001). However, pollutant remobilization from glaciers may not be a major influence on the overall POP budget in Arctic ecosystems. One exception is DDT, for which Arctic glacial melt is projected to be a significant climate-modulated source (Blais et al., 2001).

Organic carbon cycles in terrestrial and aquatic systems will also be altered by climate change, which will in turn alter POP distributions (Macdonald et al., 2003; Magnuson et al., 1997; Schindler et al., 1997). Declines in dissolved organic carbon (DOC) were observed between 1970 and 1990 in boreal lakes in northwestern Ontario during an extended period of climate warming and drought coupled with increased forest fires (Schindler et al., 1997). The declines in DOC were

attributed to decreased stream flow to lakes caused by drought and increased evaporation from warming. POPs will readily partition from water to carbon-rich particles, such as DOC. Thus, reduced DOC levels due to climate change could reduce the capacity of waters to bind these contaminants thereby making them more bioavailable for uptake by aquatic species (Magnuson et al., 1997; Schindler et al., 1997).

Temperature-induced acceleration of organic carbon metabolism by soil and sediment biota could also increase contaminant concentrations and promote partitioning to water and aquatic biota (Macdonald et al., 2003). However, biodegradation rates of POPs will also increase with rising soil and sediment temperature (Fig. 1, Legend Items H, M), which may ameliorate POP increases from this solvent depleting process (Sinkkonen and Paasivirta, 2000; Sweetman et al., 2005). Increased temperatures will also increase the volatilization of POPs from soils to air (Fig. 1, Legend Items G, K) where they will be subject to photodegradation and transport (Fig. 1, Legend Item C) (Beyer et al., 2003; Brubaker and Hites, 1998; Ma et al., 2004; Scheyer et al., 2005). For example, the loss of permafrost associated with rising temperatures will re-release pollutants from these once frozen soils making them available for atmospheric partitioning or runoff to aquatic systems (Macdonald et al., 2005).

In addition to the many abiotic factors that can influence contaminant behavior, altered species migration patterns linked to climate change could be an important factor modulating the transport of POPs (Blais et al., 2007). Migratory species, particularly fish, birds, and marine mammals, may be exposed to contaminants in one location and transport these contaminants in substantial quantities to other locations. This biotic transport of contaminants may be similar in magnitude to atmospheric and oceanic transport (Burek et al., 2008). There is evidence, for example, that Arctic and Antarctic birds may act as vectors transporting persistent contaminants from oceans to terrestrial systems via their guano (Blais et al., 2005). In Canadian coastal ponds under the nesting cliffs of northern fulmars (*Fulmarus glacialis*), concentrations of HCB, DDT, and mercury were 10 to 60 times higher than contaminant concentrations in sediments from unaffected ponds. Similar results have been observed for Antarctic seabirds, whereby elevated DDT and HCH levels have been measured in sediments at locations where penguins historically migrated (Blais et al., 2007). These studies provide some evidence that climate-induced fluctuations in the migratory patterns of birds could play an important role in altering the local and global transport of POPs (Burek et al., 2008). In addition, PCB fluxes are up to eight times higher in sub-Arctic lakes receiving the greatest sockeye salmon (*Oncorhynchus nerkus*) returns than in lakes receiving atmospheric inputs of PCBs alone (Krummel et al., 2003). Since temperature is an important controller of anadromous and freshwater fish migrations, temperature increases linked to climate change could alter POP fate through changes in fish spawning behavior (Wrona et al., 2005).

2.3. Altered fate and behavior of pesticides

Like the POPs, climate change will influence the environmental fate and behavior of pesticides by altering fundamental mechanisms of environmental partitioning primarily through mechanisms of increased volatility, wet deposition, and enhanced degradation. While additional research is needed, many pesticides may prove to be less susceptible to solvent depleting processes than POPs since they are generally less persistent and more likely to degrade with climate change. Specifically, global warming may reduce soil and aquatic concentrations of pesticides due to a combination of increased volatilization and degradation (Bailey, 2004; Benitez et al., 2006; Van den Berg et al., 1999) (Fig. 1, Legend Items G, H, I, K, M, N). Conversely, increases in the intensity and frequency of rain and storm events will promote the wet deposition of pesticides to terrestrial and aquatic systems (Bollmohr et al., 2007; Burgoa and Wauchope, 1995; Chiovarou and Siewicki, 2007; Dabrowski et al., 2002; Presley et al., 2006; Vu et al., 2006). Independent of these distribution processes, climate change may alter the frequency and amount of pesticides used as agriculture shifts in response to the rapidly changing climate (Chen and McCarl, 2001; Reilly et al., 2001, 2003).

Volatilization is a key factor in the environmental partitioning of pesticides, and global warming could lead to enhanced volatilization of pesticides relative to soil and water. Van den Berg et al. (1999) notes that volatilization processes may be responsible for the loss of up to 50% of the applied dose of a pesticide, depending on the properties of the pesticide, application technique used and environmental conditions. With this atmospheric partitioning, pesticides may be dispersed from areas of high concentrations to areas of lower concentrations, possibly exposing new populations to the toxic effects of the pesticides (Beyer et al., 2003). In addition to enhanced volatility, climate change could have an important effect on accelerating pesticide degradation (Bailey, 2004; Benitez et al., 2006; Bloomfield et al., 2006). Bailey (2004) examined residues of the pesticide isoproturon in soils over a twenty-year period and found that from 1997–2001 increased degradation in warmer soils caused pesticide concentrations to fall too low to control weed growth 30 days earlier than in years before 1997. Additionally, increased water temperature was found to increase the photodegradation rate of several phenyl-urea pesticides (Benitez et al., 2006). Given the potential increase in the loss of applied pesticides due to enhanced volatility and degradation, a compensatory increase in pesticide applications may be necessary to be efficacious against target pests. Bloomfield et al. (2006) report on the findings of the European Food Safety Authority's 2005 Scientific Panel that for every 10 °C increase in temperature, it is predicted that the half-life of pesticides in soils may decrease by 60%.

The IPCC (2007e) reports that precipitation events and extremes are very likely to become more frequent, widespread, and intense during the 21st century. Moreover, a range of climate models supports a likely increase in the intensity of typhoons and hurricanes with heavier precipitation and higher peak wind speeds (IPCC, 2007e). Precipitation scavenges gases and aerosols, with adsorbed chemical particles, from the atmosphere and deposits them to surfaces (Fig. 1, Legend Item E). As storms and rainfall events become more intense and frequent, increasing amounts of contaminants will be deposited to surfaces and lost in runoff, predominantly as pulse releases, exposing humans and wildlife to these chemicals (Fig. 1, Legend Item J) (Bollmohr et al., 2007; Burgoa and Wauchope, 1995; Chiovarou and Siewicki, 2007; Presley et al., 2006; Vu et al., 2006). Bollmohr et al. (2007) examined the exposure and toxicity of a variety of pesticides, including chlorpyrifos and endosulfan, in arthropods and fish in the Lourens River and estuary in Western Cape, South Africa. No detectable amounts of the pyrethroids cypermethrin and fenvalerate were measured in the upper Lourens River, but these pesticides were found in the estuary at levels likely to pose acute and chronic risk to aquatic life. Pesticide concentrations in a rice paddy watershed at the Sakura river basin in Japan were monitored for 3 years starting in 2002 (Vu et al., 2006). Sixteen different herbicides were detected in the stream water, and surface drainage significantly increased during rainfall events greater than 1.5 cm per day. Elevated soil moisture associated with increased precipitation could also enhance the degradation of pesticides to differentially toxic and environmentally mobile degradates (Fig. 1, Legend Item I) (Van den Berg et al., 1999). Conversely, the hydrolytic degradation of these chemicals may be limited in regions with reduced precipitation and lower soil moisture levels (Bailey, 2004; Van den Berg et al., 1999).

In terms of the links between storm intensity and chemical contamination of aquatic systems, Chiovarou and Siewicki (2007) modeled the transport and fate of the six pesticides, atrazine, carbaryl,

diquat dibromide, imidacloprid, and fipronil, in water bodies in Volusia County, Florida and Portland, Oregon under different storm intensities. Concentrations of all six contaminants were found to increase with increasing storm intensity. Consistent with these results, Presley et al. (2006) investigated pollutant and pathogen levels in New Orleans, Louisiana shortly following Hurricane Katrina. They measured soil and sediment concentrations of several contaminants, including the POP aldrin and other semi-volatile organic pollutants, as well as several metals, and found levels that exceeded U.S. EPA human health soil screening levels, which are used to identify hazardous waste sites that merit further evaluation under Superfund law. Burgoa and Wauchope (1995) also found a five-fold increase in applied pesticide loss to runoff during extreme rainfall events. These studies provide evidence that the influence of climate change on increasing storm intensity and frequency could lead to episodes of heightened contamination of water bodies and surrounding watersheds.

It is not possible to fully consider the effects of climate change on pesticide distributions in the environment without also considering anticipated shifts in agriculture. Climate change is likely to affect agriculture by shifting the location and type of crops grown and the range and magnitude of crop pests. Pesticide use will shift in response to these altered cropping patterns and crop pest distributions. Although most investigations have focused on the U.S. and Europe, growers are expected to be able to expand crop production to higher latitudes and altitudes not currently suitable for farming (Bloomfield et al., 2006; MAFF, 2000; Reilly et al., 2003; Tubiello et al., 2002).

Tubiello et al. (2002) predict that both wheat and corn production will migrate north in the U.S. due to increased temperature and precipitation, while hotter and drier climates in the south will experience decreased crop production. Warmer temperatures in northern regions will also lead to longer growing seasons, potentially allowing increased farming and increased pesticide use. Farmers will also be able to grow new crops in areas currently under cultivation with other crops. Increased temperatures may make the currently temperate south of England favorable for growing sunflower, grapes, peaches (Fuller et al., 2001), and grain maize (Bloomfield et al., 2006). These types of expanded cropping patterns will likely result in new pesticide uses on naïve ecosystems, as well as potential increases in the volume and array of pesticides used.

Another route by which climate change is likely to affect pesticide use is by altering the distribution and abundance of crop pests. Climate change may influence crop pest populations by reducing generation times and over-wintering mortality, increasing the number of generations and population growth rates, and altering crop–pest synchrony (Cannon, 1998; Olfert and Weiss, 2006; Patterson et al., 1999; Porter et al., 1991). Studies show that the main drivers of pest distribution and abundance are temperature, rainfall, and CO_2, all of which are being altered with climate change (Gutierrez et al., 2006; Porter et al., 1991; Rafoss and Saethre, 2003).

One early study modeled the potential distribution of the European corn borer (*Ostrinia nubilalis*) and found an estimated northward shift in the pest's European range of up to 1220 km with a temperature increase of 3–6 °C (Porter et al., 1991). Olfert and Weiss (2006) made a similar prediction for three pest species of beetles in Canada. In a more regionally based analysis, Gutierrez et al. (2006) examined the distribution and abundance of pink bollworm (*Pectinophora gossypiella*) in cotton in Arizona and California. Their model predicts that the bollworm is currently unlikely to reach pest status in the Central Valley of California, but that its range is likely to expand into the Central Valley with temperature increases of 1.5–2.5 °C. Rafoss and Saethre (2003) predict that the codling moth (*Cydia pomonella*) will extend its range and abundance in Norway with increasing temperatures, and that the Colorado potato beetle could migrate into Norway where it is not currently established. In contrast to the studies described above, Newman (2005) predicted that climate change would reduce the abundance of aphid species in southern Britain. These varied results demonstrate that while pests may generally increase in number and distribution, changes are likely to be species and region specific.

Some studies have examined how pesticide use could shift in response to these expected climate change-induced alterations in pest distributions and intensity (Chen and McCarl, 2001; Reilly et al., 2001, 2003). For example, Reilly et al. (2003) focus modeling on the decades of the 2030s and 2090s and assess climate change impacts on pesticide use by measuring pesticide expenditures. They project climate-linked increases in pesticide expenditures in the U.S. ranging from 10–20% on corn, 5–15% on potatoes, and 2–5% on soybeans and cotton, but variable shifts in pesticide expenditures on wheat of ±15% depending on the region and climate change scenario. No delineation is provided concerning the difference in pesticide expenditures between the decades studied. In addition, this modeling applies the IPCC's IS92A emissions scenario, which has since been updated by the IPCC under its "Special Report on Emission Scenarios" (IPCC, 2000). Despite these limitations, the results from this study are generally consistent with findings by Chen and McCarl (2001) in which increases in U.S. pesticide expenditures are projected in 2090 for corn, cotton, potatoes, and soybeans pests, with variable changes in wheat-related pesticide expenditures.

Expanded cropping patterns and increased pest pressures are expected to increase the variety and amount of pesticides used. Moreover, increased pesticide usage may be necessitated as climate change enhances chemical volatilization, degradation, and runoff. Taken together, these climate change-induced shifts in agriculture may increase human and wildlife exposures to pesticides.

3. Effects of climate change on contaminant-linked human health effects

The IPCC projects that climate change is likely to affect the health of millions of people, and that the effects will be mostly negative (Confalonieri et al., 2007). The elderly, infants, children, and urban poor are expected to be most vulnerable to the rapidly changing climate (Confalonieri et al., 2007; Ebi et al., 2006; Patz et al., 2000a, 2005). Notable adverse consequences of climate change on human health include increased death and injury associated with more severe and frequent heat waves, extreme weather events, and enhanced vector-borne and allergic disease transmission. While adverse health outcomes are projected to be greatest in low-income countries, more severe, frequent, and widespread heat waves and storm events will also impact developed countries unprepared to cope with these events (Confalonieri et al., 2007).

There continues to be a lack of data describing the effects of contaminant exposures on human health and vulnerable subpopulations under projected climate change scenarios. However, a number of studies suggest that the toxicity of ozone and PM will be exacerbated with global warming, and some of these data support that older adults will be especially vulnerable (Bell et al., 2007; Confalonieri et al., 2007; Dominici et al., 2006; Fiala et al., 2003; IPCC, 2007c; Katsouyanni et al., 1993; Knowlton et al., 2004; Koken et al., 2003; Mauzerall et al., 2005; Ordonez et al., 2005; Rainham and Smoyer-Tomic, 2003; Ren and Tong, 2006). Other potential interactions between climate change and toxicant exposure include increased susceptibility to pathogens (Abadin et al., 2007; Nagayama et al., 2007; Smialowicz et al., 2001) and aeroallergens (D'Amato et al., 2002; Diaz-Sanchez et al., 2003; Epstein, 2005; Janssen et al., 2003). Table 1 summarizes important interactions between climate change, toxicant exposures, and human health.

3.1. Vulnerable subpopulations

Elucidating the relationship between humans and the climate is complicated by the interactive nature of the many environmental,

Table 1
Climate change-induced effects of contaminants on human health.

Climate change-induced effect	Relationships/Interactions	References
Increased cardio-respiratory disease	• ↑ temperature exacerbates the adverse effects of ozone and PM • The elderly and individuals with pre-existing cardio-respiratory disease may be more vulnerable to these effects	(Bell et al., 2007; Confalonieri et al., 2007; Dominici et al., 2006; Fiala et al., 2003; IPCC, 2007a; Katsouyanni et al., 1993; Knowlton et al., 2004; Koken et al., 2003; Mauzerall et al., 2005; Ordonez et al., 2005; Rainham and Smoyer-Tomic, 2003; Ren and Tong, 2006)
Altered exposure and risk	• Some populations may experience increases or decreases in POP exposures and health risks depending on the region and diet of exposed individuals • Pesticides may impair mechanisms of temperature regulation especially during times of thermal stress	(Bard, 1999; Gordon, 1997; McKone et al., 1996; Watkinson et al., 2003)
Increased susceptibility to pathogens	• Toxicants can suppress immune function, and climate-induced shifts in disease vector range will result in novel pathogen exposure • Immune system impairment linked to toxicants may increase human vulnerability to climate shifts in pathogens • Low-income populations, infants, children, and the chronically ill may be more susceptible	(Abadin et al., 2007; Haines et al., 2006; Lipp et al., 2002; Nagayama et al., 2007; Patz et al., 2005; Rogers and Randolph, 2000; Smialowicz et al., 2001)
Increased allergenicity potential	• Air pollution and allergen exposures linked to climate change can exacerbate allergic disease and asthma incidences • Climate change enhanced allergen production coupled with POP exposures may sensitize individuals to allergic disease • Low-income populations, infants, children, and the chronically ill may be more susceptible	(D'Amato et al., 2002; Diaz-Sanchez et al., 2003; Epstein, 2005; Janssen et al., 2003)

biological, and socioeconomic conditions that can influence human health (Epstein, 2005; Haines et al., 2006; McMichael et al., 2006; Patz et al., 2005). The nature of negative health outcomes linked to climate change and the ability of populations to acclimate will depend on many conditions. These conditions include the age distribution and prevalence of inherited disease across the population, the surrounding physical and biological environment, and the many social and economic variables that influence population health (e.g., education, health care infrastructure, economic development) (Haines et al., 2006; McMichael et al., 2006).

Assessments of the U.S. population have identified the very young (<1 year), older adults (>65 years), and immuno-compromised individuals as more vulnerable to climate change because they have a reduced capacity to acclimatize to extreme heat and are also more vulnerable to vector-, food-, and water-borne disease (Ebi et al., 2006; Patz et al., 2000b). Ebi et al. (2006) note that there will be 100 million more Americans that are aged 65 or older in 2100 than in 2000, leading to generally increased vulnerability of the U.S. population to climate sensitive health outcomes. The effects of contaminants on vulnerable subpopulations warrant further study, although there is evidence that older individuals will be more susceptible to climate–air pollutant interactions (Fiala et al., 2003; Koken et al., 2003; Ordonez et al., 2005). In addition, low-income populations, infants, children, and chronically ill individuals may be especially susceptible to climate sensitive outcomes linked to interactions between pollutant exposures and changes in vector-borne and allergic disease (D'Amato et al., 2002; Diaz-Sanchez et al., 2003; Epstein, 2005; Haines et al., 2006; Janssen et al., 2003).

3.2. Air pollutants and cardio-respiratory disease

Studies examining interactions between climate change, air pollution, and human health have focused largely on tropospheric ozone and PM (Confalonieri et al., 2007; IPCC, 2007c). Generally, heat appears to render people more vulnerable to the adverse effects of air pollution. Climate change-induced increases in tropospheric ozone and PM, as is projected for many regions, coupled with global warming may exacerbate human vulnerability to cardio-respiratory disease especially among older adults.

Rising temperatures appear to increase susceptibility to cardio-respiratory disease linked to air pollution exposures. Epidemiological evidence suggests that heat exacerbates mortality and morbidity from cardio-respiratory disease in humans exposed to ozone and PM (Fiala et al., 2003; IPCC, 2007c; Koken et al., 2003; Ordonez et al., 2005; Rainham and Smoyer-Tomic, 2003). During the European heat wave of 2003, there was a surge in respiratory illnesses that was associated with increased concentrations of particulates and ozone especially among the elderly (Fiala et al., 2003; Ordonez et al., 2005). In another study illustrating the effects of climate sensitive outcomes on vulnerable older populations, males in Denver, Colorado aged 65 and older were found to be at increased risk for hospitalization for acute myocardial infarction, coronary arteriosclerosis, and pulmonary heart disease when co-exposed to higher temperatures and ozone (Koken et al., 2003). More recently, Bell et al. (2008) examined confounding factors, including air pollution levels, on heat-related mortality in three Latin American cities: Mexico City, Mexico, Sao Paulo, Brazil, and Santiago, Chile. They found that ozone and PM_{10} enhanced heat-related mortality, and that susceptibility was associated with increasing age in all three cities.

Modeling studies also show increased mortality and morbidity with increased ozone exposure coupled with global warming (Bell et al., 2007; Knowlton et al., 2004; Mauzerall et al., 2005; Rainham and Smoyer-Tomic, 2003). For example, using the IPCC A2 climate scenario (i.e., high growth of CO_2), a 4.5% increase in ozone-related deaths in the U.S. from climate change was modeled for the mid 2050s compared to the 1990s (Knowlton et al., 2004). Similarly, Bell et al. (2007) estimated elevated ozone in 50 U.S. cities applying the IPCC A2 climate scenario and found a corresponding increase in daily total mortality of 0.11% to 0.27%. By examining cardio-respiratory mortality in Toronto, Canada from 1980 to 1996, Rainham et al. (2003) detected a small, but consistent effect of air pollution (ozone, NOx, SO_2, CO, and PM_{10}) on temperature/humidity-related mortality. Recently, Ren et al. (2008) modeled the modulating effects of temperature and ozone interactions on mortality from 1987 to 2000 in 60 large eastern U.S. cities, and found that temperature had a synergistic effect on ozone-related mortality in the northeast. Specifically, for each 10 ppb increase in ozone, low, medium, and high temperatures increased mortality by 2.22%, 3.06%, and 6.22%, respectively. However, in the southeast U.S., the effects of temperature on ozone mortality were less robust than in the northeast. This suggests that regional differences (e.g., geography, population age structure, culture) may contribute to altering the effects of climate change and air pollution on adverse health outcomes.

Increasing temperatures may also modify the associations between PM and cardio-respiratory disease. Qian et al. (2008) found a synergistic effect of PM_{10} and high temperatures on daily cardio-respiratory

mortality in Wuhan, China. The PM_{10} effects were strongest on extremely high temperature days (daily average temperature 33.1 °C) and weakest during normal temperature days (daily average temperature 18 °C). Epidemiological data collected in Brisbane, Australia from 1996 to 2001 shows that respiratory- and cardiovascular-related hospital admissions and mortality were elevated when both temperature and PM concentrations increased (Ren and Tong, 2006). Katsouyanni et al. (1993) examined the interaction of smoke (PM), SO_2, and ozone with deaths in Athens, Greece during a July 1987 heat wave versus deaths in the 6 previous Julys (1981–1986). They found a significant positive association between SO_2 concentrations and temperature on the number of deaths when the average daily temperature was at or above a threshold of 30 °C. Dominici et al. (2006) constructed a database of hospital admission rates from 1999 to 2002 using U.S. Medicare data for cardiovascular and respiratory outcomes and injuries, ambient $PM_{2.5}$ concentrations, and temperature. They identified an association between $PM_{2.5}$ and hospital admission rates for respiratory outcomes that was positively correlated with temperature. Moreover, a comparison of regions with average temperatures that differed by 1 °C showed that the warmer regions had an additional nine hospital admissions per 10,000 individuals for respiratory tract infections per 10 $\mu g/m^3$ increase in $PM_{2.5}$. In contrast to the body of evidence showing positive associations between temperature and air pollution on death and disease, Samet et al. (1998) found little relationship between temperature and particulate matter on mortality upon examination of mortality data for Philadelphia, U.S. from 1973–1980.

3.3. *Altered effects of POPs and pesticides*

Questions concerning climate change impacts on the toxicity and risks to humans exposed to POPs and pesticides has received scant attention. McKone et al. (1996) conducted a study to model the effects of a 5 °C increase in temperature on human health risks in western U.S. populations exposed to HCB. Their analysis concluded that this global warming scenario would have little negative impact on health risk associated with HCB among these populations. In fact, exposures to humans might decline because of enhanced environmental degradation and the tendency of HCB to partition to the atmosphere with rising temperature (Ma et al., 2004). This atmospheric partitioning would remove it from water, thereby reducing exposures to aquatic biota, and in turn, potentially reducing human dietary exposures (Macdonald et al., 2005). However, under this scenario, this compound could then be subject to atmospheric transport to northern latitudes, where wet deposition to aquatic systems may lead to potentially elevated dietary exposures and health risks among exposed northern and indigenous communities (Bard, 1999).

Chemical toxicant exposures may also affect homeostatic temperature regulation in humans and other endotherms. Organophosphate and carbamate insecticides are known to elicit a fever in humans. Conversely, acute exposures in the rat lead to an acute reduction in core temperature followed by a delayed elevation in the core temperature (Gordon, 1997; Watkinson et al., 2003). In additional experiments, rats have been chronically exposed to dietary chlorpyrifos, and then subsequently challenged with a larger dose of chlorpyrifos (Gordon and Padnos, 2002). The ensuing hypothermic response was observed to be greater than for a normal acute dose, indicating that chronic exposure may sensitize the thermoregulatory response. Intoxication by these classes of pesticides may make it even more difficult for humans (and other endotherms) to maintain normal core temperatures, especially during times of thermal stress, such as heat waves.

3.4. *Increased vulnerability to disease vectors*

The potential for adverse human health impacts extends beyond those direct effects linking climate change to augmented exposures and toxicity. Climate change-induced shifts in disease vector range and severity coupled with contaminant exposures could increase human vulnerability to disease by impinging on the ability of individuals to mount an effective immune response to new pathogen exposures.

The distribution and emergence of vector-borne diseases, such as malaria and cholera, are predicted to be dependent on temperature, humidity, and precipitation (Lipp et al., 2002; Patz et al., 1996, 2005; Rogers and Randolph, 2000). As such, climate change is predicted to facilitate the reemergence or expansion of endemic vector-borne diseases or might promote the migration of these diseases to new regions. For example, cholera incidences in south Asia are linked to weather patterns (Patz et al., 2000a) and are predicted to increase with shifts in precipitation patterns (IPCC, 2007c). Likewise, malaria is predicted to migrate into higher latitudes and altitudes, particularly in Africa and South America, where it is endemic, although regions of Africa are also predicted to see declines due to high temperatures and desertification (IPCC, 2007b).

Evidence supports a link between contaminant exposures and suppressed immune system function (Abadin et al., 2007; Nagayama et al., 2007; Smialowicz et al., 2001). Immunotoxicity is a sensitive endpoint for several POPs, including heptachlor, PCBs, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Exposures to POPs may decrease the ability of humans (and other animals) to fight infection (Abadin et al., 2007). Young rats exposed to heptachlor were observed to have suppressed antibody-mediated immune response as adults (Smialowicz et al., 2001). A study of Japanese infants found that perinatal exposure to dioxins, PCBs, and organochlorine pesticides altered the ratios of lymphocyte subsets, potentially leading to increased autoimmune disease and immune suppression later in life (Nagayama et al., 2007). While further study is need, immune system impairment linked to toxicant exposures may increase human vulnerability to climate-induced shifts in vector borne and infectious diseases. Populations living in lower income countries may be especially vulnerable to these pathogen–pollutant interactions as they may lack the resources to prevent and manage disease (Haines et al., 2006).

3.5. *Allergenicity*

In addition to changes in vector-borne disease, the incidences and severity of allergic disease are rising, especially in industrialized countries (D'Amato et al., 2002; Diaz-Sanchez et al., 2003). Asthma prevalence has quadrupled in the United States since 1980. Air pollution and higher concentrations of CO_2-induced allergens linked to climate change may be contributing to increased rates of allergic disease and asthma (Epstein, 2005; Shea et al., 2008).

Shifts in plant populations have already been documented as a result of climate change (Rogers et al., 2006; Root et al., 2003; Singer et al., 2005). For example, studies show that increasing concentrations of CO_2 enhance the production of Amb a 1 allergen and pollen from ragweed (*Ambrosia artemisiifolia* L.) (Rogers et al., 2006; Singer et al., 2005). In addition, diesel exhaust, which contains numerous pollutants, including PM, NOx, VOCs, CO, and polycyclic aromatic hydrocarbons (PAHs), has been shown to enhance allergenicity and asthma symptoms in adults and children by acting synergistically with allergens (D'Amato et al., 2002; Diaz-Sanchez et al., 2003; Janssen et al., 2003). For example, Janssen et al. (2003) found that Dutch children aged 7–12 from 24 schools within 400 m of a major roadway had increased sensitization to outdoor allergens. This relationship between adverse symptoms and traffic-related air pollution was largely restricted to children with pre-existing bronchial hyperresponsiveness (common among asthmatics) and allergen sensitivity. Thus, the combination of enhanced air pollution and allergen production linked to climate change may exacerbate allergic disease and asthma incidences in vulnerable individuals, especially children, infants, and asthmatics (Epstein, 2005).

4. Effects of climate change on contaminant toxicity to wildlife

There is substantial evidence that climate change is affecting the phenology of organisms, the range and distribution of species, and the composition and dynamics of communities (Lovejoy and Hannah, 2005; Penuelas and Filella, 2001; Root et al., 2003; Walther et al., 2001). While species have historically acclimated or adapted to changes in climate, the rapid rate of current climate change coupled with increasingly fragmented and impaired habitats present unprecedented challenges for modern species (Boone et al., 2007; Fisk et al., 2005; Occhipinti-Ambrogi, 2007; Rohr et al., 2004; Schiedek et al., 2007).

The bioavailability and toxicity of POPs and pesticides in wildlife is likely to increase in response to rising temperatures and salinity (Boone and Bridges, 1999; Capkin et al., 2006; Gaunt and Barker, 2000; Heugens et al., 2001; Moore et al., 2003; Schiedek et al., 2007; Silbergeld, 1973; Song and Brown, 1998; Tachikawa and Sawamura, 1994; Wang et al., 2001; Waring and Moore, 2004). An underlying mechanism of this interactive toxicity is that temperature alters the toxicokinetics of chemical pollutants in exposed biota (Buchwalter et al., 2003; Lydy et al., 1999; Maruya et al., 2005). Another mechanism probably influencing this enhanced toxicity is that increasing temperature can alter homeostasis and other key physiological mechanisms, thereby exacerbating the adverse effects of contaminants (Anderson and Peterson, 1969; Broomhall, 2002, 2004; Gordon, 2003; Heath et al., 1994; Patra et al., 2007).

Some populations, particularly those living at the edge of their homeostatic or physiological tolerance range, may be more vulnerable to the to the dual stresses of climate change and contaminant exposures (Anderson and Peterson, 1969; Gordon, 2003; Heath et al., 1994; Patra et al., 2007). Moreover, the rapidity of climate change-induced shifts in habitats and trophic food webs could affect contaminant toxicity by altering exposure pathways and increasing susceptibility of some populations, especially those already under stress (AMAP, 2004; Breivik et al., 2004; Brook and Richardson, 2002; Gaston et al., 2003; Gilbertson et al., 2003; Macdonald et al., 2005; Olafsdottir et al., 1998; Sagerup et al., 2000). A limitation of studies investigating the interactive toxicity of climate change and contaminant exposures is that observed biological effects may prove to have a non-linear relationship to the stressors. That is, an incremental increase in temperature or contaminant may be less important than thresholds or tipping points that trigger potentially major synergisms in adverse effects across species, populations, and communities. Table 2 summarizes important climate change–contaminant interactions in wildlife.

4.1. Altered uptake and elimination

Increasing temperatures will generally increase the uptake and excretion of toxicants. For example, Buchwalter et al. (2003) observed enhanced uptake of the organophosphate (OP) pesticide chlorpyrifos with increasing temperatures among three aquatic insect species: *Notonecta kirvyi*, *Pteronarcys californica*, and *Dicosmoecus gilvipes*. Likewise, uptake of the pesticides chlorpyrifos and methyl-parathion, and the POP pentachlorobenzene, increased at 20 °C and 30 °C compared to 10 °C in the midge, *Chironomus tentans* (Lydy et al., 1999). Decreased body burdens of chlorpyrifos and methyl-parathion were also observed at higher temperatures, indicating increased metabolism and excretion. Yet, body burdens did not change for pentachlorobenzene at any of the three temperatures tested. In the estuarine fish, *Fundulus heteroclitus*, warmer temperatures (25 °C) contributed to a rate of elimination of toxaphene congeners that was two-fold higher than in cooler water (15 °C) (Maruya et al., 2005). Similarly, Paterson et al. (2007) monitored elimination of 72 PCB congeners in perch under typical northern latitude annual temperature cycles, and found that elimination occurred only during the spring and summer months when water temperatures were near or above 20 °C.

4.2. Increased toxicity

The toxicity of contaminants may be enhanced with increasing temperatures (Boone and Bridges, 1999; Capkin et al., 2006; Gaunt and Barker, 2000; Silbergeld, 1973). While the exact mechanisms underlying this relationship are not fully understood and the majority of research focuses on aquatic species, studies indicate that temperature-induced shifts in metabolism are one controlling factor (Buckman et al., 2007; Lydy et al., 1999; Monserrat and Bianchini, 1995).

The lethality of the POP dieldrin to the freshwater darter (*Etheostoma nigrum*) increased with increasing temperatures (Silbergeld, 1973). In the green frog (*Rana clamitans*), the toxicity of the insecticide carbaryl increased with temperature increases from 17 °C to 22 °C to 27 °C (Boone and Bridges, 1999). Gaunt and Barker (2000) found that the toxicity of the herbicide atrazine to catfish (*Ictalurus punctatus*) increased with increasing temperature or decreasing dissolved oxygen. They predicted that changes in these two parameters, which would likely occur simultaneously in climate change scenarios, could greatly enhance the toxicity of atrazine to some aquatic species. Capkin et al. (2006) observed increased mortality in juvenile rainbow trout (*Oncorhynchus*

Table 2
Climate change-induced toxicological effects of contaminants on wildlife.

Climate change-induced effect	Relationships/Interactions	References
Altered uptake and elimination	• ↑ temperature = ↑ uptake of toxicants • ↑ temperature = ↑ elimination • ↑ temperature = remobilization of bioaccumulated POPs	(Buchwalter et al., 2003; Lydy et al., 1999; Maruya et al., 2005)
Increased toxicity	• ↑ temperature = ↑ toxicity • ↑ temperature = ↑ metabolism and potentially altered metabolite profiles • Toxicant exposure may limit capacity of species and populations to acclimate to altered temperatures. • Pollutant-exposed ectotherms and species at the edge of their physiological tolerance range may be especially sensitive to temperature increases.	(Anderson and Peterson, 1969; Boone and Bridges, 1999; Brian et al., 2008; Broomhall, 2002, 2004; Buckman et al., 2007; Capkin et al., 2006; Gaunt and Barker, 2000; Gordon, 2003; Heath et al., 1994; Lydy et al., 1999; Monserrat and Bianchini, 1995; Patra et al., 2007; Silbergeld, 1973)
Altered environmental salinity	• ↓ solubility and ↑ bioavailability of pesticides/POPs ("salting out effect") • ↑ salinity + ↑ POP/pesticide exposure may alter osmoregulation due to altered enzymatic pathways	(Fortin et al., 2008; Heugens et al., 2001; Moore et al., 2003; Schiedek et al., 2007; Schlenk and El-Alfy, 1998; Schwarzenbach et al., 2003; Song and Brown, 1998; Tachikawa and Sawamura, 1994; Wang et al., 2001; Waring and Moore, 2004)
Altered ecosystems	• Altered POP sequestration and/or remobilization through shifts in food sources and starvation events • Shifts in disease vector range and severity coupled with toxicant exposure inhibiting immune response may leave wildlife more susceptible to disease • Low level exposures may impair organism acclimation to ecosystem alterations induced by climate change • Climate change induced changes in trophic food webs may alter POP bioaccumulation and biomagnification	(AMAP, 2004; Braune et al., 2005; Furnell and Schweinsburg, 1984; Jenssen, 2006; Macdonald et al., 2005, 2003; Olafsdottir et al., 1998; Ramsay and Stirling, 1982; Schiedek et al., 2007, Stirling et al., 1999)

mykiss) exposed to the insecticide endosulfan as temperature was increased from 13 °C to 16 °C. In contrast to these findings, pyrethroids and DDT are generally thought to be more toxic under low temperature conditions, which may be due to a sodium channel modulated increase in nervous system vulnerability at lower temperatures (Narahashi, 2000). However, others have observed increased pyrethroid toxicity at elevated temperatures in leopard frogs (*Rana* spp.) (Materna et al., 1995) and water fleas (*Daphnia magna*) (Ratushnyak et al., 2005), illustrating the species-specific response to increased temperatures and toxicant exposures.

Temperature-dependent changes in metabolism appear to be one important mechanism modulating the biotransformation and enhanced toxicity observed under elevated temperature conditions. For example, despite the relatively high persistence of POPs in biota, Buckman et al. (2007) observed enhanced biotransformation of PCBs to the toxicologically active hydroxylated PCB metabolites by rainbow trout with rising temperature (8, 12, and 16 °C). Moreover, Lydy et al. (1999) postulate that while body burdens of the OP insecticides decline at higher temperatures, toxicity is ultimately enhanced due to an acceleration of the biotransformation of the OP insecticides to their more toxic *ortho*-analog metabolites. Monserrat and Bianchini (1995) suggested a similar explanation for the increased toxicity they observed when exposing crabs (*Chasmagnathus granulata*) to methyl parathion. There was an approximately ten-fold increase in acute lethality with temperature change from 12 °C to 30 °C. The authors suggest that the higher temperature favors enzymatic activation of the organophosphate over degradation and excretion.

The metabolism studies demonstrate a general concept that is likely to hold true for the effect of temperature on toxicity of many contaminants. While the rates of uptake and excretion may generally increase with increasing temperature, the ultimate toxicity of these contaminants will depend on whether changes in metabolism result in increased bio-activation or detoxification.

4.3. Altered homeostasis and physiological responses

The ability of species and populations to tolerate elevated temperatures may be impaired with toxicant co-exposures. Alterations in climate change parameters, predominantly temperature, will act as co-stressors with chemical toxicants, thereby affecting physiological processes and the ability of wildlife to maintain homeostasis (Broomhall, 2004). Ectotherms, such as fishes, amphibians, and reptiles, may be particularly vulnerable to these temperature–contaminant interactions. Moreover, species living at the edge of their physiological tolerance range may be less able to cope with the dual stressors of climate change and contaminant exposures (Anderson and Peterson, 1969; Gordon, 2003; Heath et al., 1994; Patra et al., 2007).

The generalized stress of maintaining homeostasis under increasing temperatures may potentiate the effects of some pesticides. When eggs of the Australian frog (*Limnodynastes peronii*) were reared under a high and low temperature regimen and exposed to the insecticide endosulfan, there was a negative effect of endosulfan on predator avoidance that was proportionally worse for the tadpoles reared at a higher temperature (Broomhall, 2004). This same effect was observed in a previous study with another amphibian species, *Litoria citropa* (Broomhall, 2002). Upper temperature tolerance limits were also reduced in the following four species of freshwater fish exposed to endosulfan and chlorpyrifos: silver perch (*Bidyanus bidyanus*), eastern rainbow fish (*Melanotaenia duboulayi*), western carp gudgeon (*Hypseleotris klunzingeri*), and rainbow trout (Patra et al., 2007). The ability of brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) to acclimate to increasing temperature is impaired by sub-lethal doses of DDT (Anderson and Peterson, 1969). Heath et al. (1994) found that exposure of fathead minnows (*Pimephales promelas*) to low doses of the pyrethroid insecticide cyfluthrin could reduce their zone of temperature tolerance by 30%. Cyfluthrin exposure caused a maximum decrease of 3.3 °C below median heat tolerance levels and a 5.6 °C increase in median cold tolerance levels. They observed effects at concentrations as low as 170 parts per trillion.

Another important consideration for climate change and pollutant interactions is the timing of exposures at sensitive life stages inducing responses that in turn alter physiological processes. Brian et al. (2008) measured a transient increase of the yolk precursor protein, vitellogenin (VTG), in male fathead minnows at higher temperatures (30 °C vs. 20 °C) upon exposure to a mixture of endogenous steroidal estrogen, 17β-estradiol, synthetic steroidal estrogen, 17α-ethinylestradiol, and other estrogenic chemicals (4-tertnonylphenol, 4-tertoctylphenol, and bisphenol-A). The temperature-dependent increase in VTG was observed only during the first 24 h of exposure, demonstrating that the effects of elevated temperature were more pronounced early in the exposure period. Increased storm intensity and frequency associated with climate change could lead to episodes of high contaminant exposures due to chemical runoff. High exposure episodes that coincide with sensitive life stages, such as during maturation, spawning, and development, may be detrimental to aquatic species fitness and survival.

4.4. Altered environmental salinity

In addition to global warming, climate change-induced shifts in precipitation and evaporation patterns have resulted in increased salinity in subtropical and tropical oceans and a freshening of mid and high latitude waters (IPCC, 2007e). Sea level rise linked to climate change is projected to lead to salt water intrusion into previously freshwater habitats (IPCC, 2007e). However, salinity could decrease in waters receiving elevated inputs of freshwater due to increases in precipitation or snow and ice melt. In sum, the effects of climate change on salinity patterns are complex and may vary by region as a number of factors can influence this parameter. For example, in brackish water ecosystems, like the Chesapeake Bay, salinity patterns contribute to species distributions and are predicted to shift in response to climate change (Pyke et al., 2008; Rogers and McCarty, 2000). Salinity reductions are expected during winter due to projected increases in tributary flow linked to elevated precipitation. Conversely, increased regional drought frequency and sea level rise are predicted to lead to saltwater intrusion events and elevated salinity for portions of the Bay (Pyke et al., 2008; Rogers and McCarty, 2000).

Salinity–contaminant interactions are made additionally complex because salinity can influence the chemical itself or it may modulate toxicity and physiological functioning of species (Fortin et al., 2008; Heugens et al., 2001; Moore et al., 2003; Schiedek et al., 2007; Schlenk and El-Alfy, 1998; Schwarzenbach et al., 2003; Song and Brown, 1998; Tachikawa and Sawamura, 1994; Wang et al., 2001; Waring and Moore, 2004). Organic compounds are generally less soluble and more bioavailable in saltwater than in freshwater due to the "salting out" effect whereby water molecules are strongly bound by salts making them unavailable for dissolution of organic chemicals (Schwarzenbach et al., 2003). Thus, increased contaminant bioavailability and toxicity is possible in subtropical latitudes experiencing increased salinity, as well as in estuaries and coastal freshwater ecosystems subject to increased saltwater intrusion or droughts. Consistent with this hypothesis, increased mortality to the organophosphate pesticide dimethoate was observed in salt marsh mosquitoes (*Aedes taeniorhynchus*) and brine shrimp (*Artemia* sp.) under hyperosmotic conditions (i.e., 3–4 times the isoosmotic salinity) (Song and Brown, 1998). The authors concluded that the increased toxicity might be attributable to increased dimethoate bioavailability and accumulation at the elevated salinity levels compared to the isoosmotic conditions. They also report that another organophosphate pesticide, malathion, has a higher degradation half-life in seawater (3–5 days) than in freshwater (1 day), supporting the idea of higher persistence due to salting out effects.

Heugens et al. (2001) attribute the increased toxicity observed at elevated salinity to higher physiological costs for organisms to maintain osmoregulation leading to a decline in fitness and elevated sensitivity to contaminant exposures. There is support for this assertion as several studies show that altered salinity profiles coupled with POP and pesticide exposures may alter osmoregulatory function in aquatic organisms (Fortin et al., 2008; Hall et al., 1995; Moore et al., 2003; Schiedek et al., 2007; Schlenk and El-Alfy, 1998; Tachikawa and Sawamura, 1994; Wang et al., 2001; Waring and Moore, 2004).

Spikes in atrazine concentrations may occur after heavy rain events with concentrations reported in North American rivers at up to 108 μg/L and in the Chesapeake Bay at up to 30 μg/L (Fortin et al., 2008). A 96-hour exposure to atrazine at 5 μg/L impaired osmotic control in *F. heteroclitus* larvae, with higher prevalence of dehydrated larvae at isoosmotic (15 ppt) and extreme (35 ppt) salinities and hyperhydrated larvae at low salinities of 3 ppt (Fortin et al., 2008). In the absence of atrazine, salinity had no effect on the prevalence of hyper or hypo hydrated fish. In estuarine copepods (*E. affinis*), high (25 ppt) and low (5 ppt) salinity levels increase mortality in response to high doses of the atrazine (>2.6 mg/L) (Hall et al., 1995). The authors concluded that *Eurytorma* might be more physiologically effective at metabolizing atrazine at intermediate salinities, although impaired osmotic control at these salinity extremes is probably an important contributor to the elevated mortality. Similar results were observed in another study exposing the copepod, *Microarthridion littorale* to chlorpyrifos and DDT (Staton et al., 2002). While the mechanism leading to the impaired osmotic control in fish and altered toxicity in copepods is unknown, alterations in enzymatic pathways have been observed in fish under similar exposures (Moore et al., 2003; Tachikawa and Sawamura, 1994; Waring and Moore, 2004).

In Japanese medaka (*Oryzias latipes*), co-exposure to the pesticide pentachlorophenol (PCP) and elevated salinity resulted in reductions in PCP uptake and increases in clearance (Tachikawa and Sawamura, 1994). Decreased uptake of PCP was associated with decreased water flux across the gills and increased clearance was linked to increased Na^+, K^+-ATPase activity and developing chloride cells. However, pre-exposing Atlantic salmon smolts to atrazine in freshwater at concentrations greater than 1.0 μg/L resulted in mortality upon a 24-hour seawater challenge (Waring and Moore, 2004). Enhanced activity of flavin-containing monooxygenases (FMOs) is another enzymatic pathway that may play a role in potentiating the toxicity of some chemical toxicants under conditions of elevated salinity (Schlenk, 1998). FMOs are induced in the presence of salinity and play a role in maintaining cellular osmotic pressure. Elevated salinity leads to increased FMO activity, which in turn enhances production of a more bioactive metabolite in aldicarb-exposed fish (Wang et al., 2001).

4.5. Altered ecosystems

There is substantial evidence of the ecological impacts of climate change across terrestrial and aquatic environments ranging from polar to tropical regions (Lovejoy and Hannah, 2005; Penuelas and Filella, 2001; Root et al., 2003; Walther et al., 2001). While some species and populations may be especially vulnerable to climate change, it is important to recognize that these impacts will be concomitant with and in some cases exacerbated by other ecosystem stressors, notably chemical pollution, invasive species, over-harvesting, habitat destruction, and pathogens. The superimposition of these increasingly common ecosystem stressors with the rapidly changing climate could further hinder wildlife acclimation and adaptation to climate change (Cook et al., 1998; Fischlin et al., 2007; Macdonald et al., 2005; Occhipinti-Ambrogi, 2007; Scavia et al., 2002). The IPCC projects that ecosystem resilience in many regions is likely to be exceeded this century by an unprecedented combination of climate change disturbances and these many other anthropogenic and natural stressors (Fischlin et al., 2007).

Climate change producing alterations in trophic structures, food sources, migratory patterns, and feeding behavior may influence processes of bioaccumulation and biomagnification in POP-exposed animals (AMAP, 2004; Furnell and Schweinsburg, 1984; Macdonald et al., 2005; Olafsdottir et al., 1998; Ramsay and Stirling, 1982; Stirling et al., 1999). Important solvent switching and solvent depletion processes involve the partitioning of POPs from water to phytoplankton and zooplankton at the base of aquatic food chains followed by bioaccumulation and biomagnification up the food chain (Braune et al., 2005; Macdonald et al., 2005). Apex predators at the top of some food webs may experience significant biomagnification of POPs as a result of these solvent switching and solvent depletion processes. For example, polar bears (*Ursus maritimus*) generally have the highest concentrations of POPs of any Arctic animal (Braune et al., 2005). Stirling et al. (1999) observe that the loss of stable ice flows linked to climate warming are the major factor contributing to Hudson Bay polar bears coming ashore for several months of fasting in progressively poorer condition. Hudson Bay polar bears prey primarily on ringed seals (*Phoca hispida*), the population of which is in decline due to a loss of these stable ice flows (Furnell and Schweinsburg, 1984; Ramsay and Stirling, 1982; Stirling et al., 1999). Polar bears near starvation will use stored fat as an energy source, remobilizing POPs sequestered in these tissues and potentially resulting in the dual stresses of starvation and chemical toxicity (Macdonald et al., 2005).

Climate change-induced POP remobilization scenarios may apply to other species as well, such as migratory salmon, common eider (*Somateria mollissima*), thick-billed murres (*Uria lomvia*), and Arctic Char (*Salvelinus alpinus*) (AMAP, 2004; Macdonald et al., 2005; Olafsdottir et al., 1998). For example, Arctic cod (*Boreogadus saida*) are a primary, high fat forage fish for many Arctic species, and loss of critical sea ice habitat may adversely affect Arctic cod populations and those animals that rely on them for food. Gaston et al. (2003) analyzed the diets of thick-billed murres from 1981–2002, and observed a decrease in consumption of Arctic cod. This shift in diet increased the fat burned to the fat energy gained. These types of shifts in food sources could lead to greater relative biological burdens and remobilization of POPs. However, POP bioaccumulation may be reduced in some predators if they are able to switch to less contaminated food sources (Brook and Richardson, 2002; Macdonald et al., 2005).

Similar to humans, climate change-induced shifts in pathogen and disease vector ranges coupled with toxic contaminant exposures could render wildlife more susceptible to disease by inhibiting their ability to mount an effective immune response (Breivik et al., 2004; Burek et al., 2008; de Swart et al., 1996; Gilbertson et al., 2003; Kajiwara et al., 2002; Sagerup et al., 2000). Glaucous gulls (*Larus argentatus*) had a higher parasitic nematode infection level that was correlated with PCB and organochlorine pesticide concentrations (Sagerup et al., 2000). In laboratory and field studies, northern leopard frogs (*Rana pipiens*) exhibited immune suppression because of DDT or dieldrin exposure (Gilbertson et al., 2003). Harbor porpoises (*Phocoena phocoena*) exhibited a significant correlation between concentrations of PCBs, polybrominated diphenyl ethers (PBDEs), toxaphene, DDT and its metabolites and thymic atrophy and splenic depletion (Breivik et al., 2004). Harbor seals (*Phoca vitulina*) fed POP-contaminated fish collected from the Bering Sea for 2.5 years had higher body burdens of POPs than seals fed relatively uncontaminated fish, and displayed impaired immune responses including suppression of natural killer cell and specific T-cell activity, (de Swart et al., 1996).

POPs, especially PCBs, DDT, dioxins, and furans, have also been investigated as cofactors contributing to recent mass mortality incidences attributed to morbilliviruses among several marine mammal populations (Burek et al., 2008; Kajiwara et al., 2002; Kuiken et al., 2006). For example, a mass mortality incident of 10,000 Caspian seals (*Phoco caspica*) in the spring and summer 2000 was attributed primarily to canine distemper virus, and like other incidences, was preceded by an unusually mild winter. Kajiwara et al. (2002) found

that POP levels, especially for DDT/DDE and PCBs, were higher in animals collected during the Caspian seal epizootic incident than in earlier collections of healthy individuals, suggesting that these contaminants made animals more susceptible to disease. More recently, however, Kuiken et al. (2006) re-analyzed tissue from the same incident but eliminated some specimens from evaluation because they were diagnosed negative for the virus. After this adjustment, the authors found that POP concentrations in diseased seals in 2000 were comparable to concentrations found in seals sampled in previous years. These mixed results are indicative of the need to better understand the interactions of POP body burdens, immune system suppression, and climate-induced changes in pathogenic disease transmission among exposed populations.

In addition to a potentially diminished immune response, other toxic effects linked to chronic, low level POP exposures may impair organism acclimation to ecosystem alterations (Jenssen, 2006). High blood levels of POPs, including HCB, oxychlordane, DDT metabolites, and PCBs, have been associated with a decrease in viable offspring, a decrease in adult yearly survival rate, and an increase in wing feather asymmetry (Bustnes et al., 2002, 2003; Kuenzel, 2003; Leeson and Walsh, 2004). Thyroid hormone deficits during early life stages affect neurodevelopment and subsequent behavior and cognitive ability in vertebrates (Donahue et al., 2004; Jenssen, 2006). Studies of polar bears have shown disrupted thyroid hormone homeostasis induced by POP exposures (Norstrom, 2000; Skaare et al., 2001; Wiig, 1995). Jenssen (2006) hypothesize that since hunting and survival skills are dependent upon behavioral and cognitive abilities, altered thyroid hormone homeostasis associated with POP exposures may be a factor hindering polar bear acclimatization to retreating sea ice. In another example, PCB levels in the glaucous gull (*Larus hyberboreus*), a top predator in the Arctic food web, were significantly related to the proportion of time that adult gulls were absent from the nest (Bustnes et al., 2001). The authors suggested that the gulls required more time to gather food as a result of endocrine disruption or neurological disorders due to high contamination levels.

Climate change may also alter patterns of POP bioaccumulation and biomagnification by altering bottom-up or top-down mechanisms controlling trophic food webs (Braune et al., 2005; Macdonald et al., 2003; Schiedek et al., 2007). Climate change-induced alterations in bottom-up controlling mechanisms, such as altered nutrient and primary production, may lead to the addition or removal of trophic levels (Macdonald et al., 2003). This in turn could shift predators higher or lower in the aquatic food web, leading to a respective increase or reduction of POPs. Top-down alterations in trophic structures elicited by the changing climate, for example, could involve the loss or diminished populations of higher trophic level species leading to consumption further down the food chain and reduced POP biomagnification potential.

5. Conclusions

There is a growing body of evidence that climate change will have broad negative impacts on the distribution and toxicity of environmental contaminants (Bell et al., 2007; Buckman et al., 2007; Confalonieri et al., 2007; Dentener et al., 2006; Fiala et al., 2003; Hogrefe et al., 2004; Knowlton et al., 2004; Macdonald et al., 2005; Patra et al., 2007; Schiedek et al., 2007; Stevenson et al., 2006). However, many areas merit further examination. Direct investigation of climate change impacts on contaminant behavior and toxicity is needed as much of the current literature examines this issue indirectly (e.g., focusing on temperature, salinity, etc.). Research that does focus on climate change directly is of great benefit, but has dealt mainly with predicting pollutant behavior under different climate change scenarios. Less work has been undertaken to describe the toxicological consequences of these altered pollutant distribution patterns. This review also underscores the lack of data describing the effects of climate change and toxicant exposures on human health. While climate change is a global phenomenon, the existing literature has only recently started to explore contaminant interactions outside of North America and Europe (e.g., Bell et al., 2008; Qian et al., 2008). A greater understanding of the biological effects of climate change on chemical toxicity continues to be needed in other parts of the world. This data gap is of special concern since impoverished populations may be particularly susceptible to the interactive effects of climate change and contaminant exposures, as these groups are often exposed to other stressors, such as malnourishment and disease.

Air pollutant concentrations are closely intertwined with climate change, making ozone and PM particularly relevant, as they are influenced by and act on climate change. Air pollution is projected to increase in many regions due to climate change, especially in areas that are urbanized, polluted, and subject to reduced precipitation and stagnant atmospheric circulation patterns. A growing body of epidemiological and modeling evidence supports that global warming coupled with ozone and PM exposures could exacerbate the prevalence and severity of human cardio-respiratory disease and mortality. Given the large segment of the population exposed to outdoor air pollutants, a relatively modest increase in mortality and morbidity estimated from current modeling projections could translate into a substantial number of individuals at risk (Patz et al., 2005; Zhang et al., 2006). Certain subpopulations, especially the elderly, infants and children, and individuals with pre-existing health conditions, such as chronic cardiopulmonary and immunological diseases, may be especially susceptible to these adverse interactions (Ebi et al., 2006; Oberdörster, 2001; Patz et al., 2000a; Pope, 2000). There continues to be uncertainty, however, including that the modeling is based usually on a single emissions scenario and other co-stressors may obscure the interactions between climate, air pollution, and human health. Given the potentially serious consequences of climate–air pollutant interactions on human health, additional research is needed to further refine the modeling projections and describe underlying mechanisms of toxicity.

For POPs and other pesticides, increases in temperature and precipitation will influence environmental distribution through increases in chemical volatility and degradation. Climate change could facilitate a number of solvent depletion processes, including ice and snow melt, altered trophic structures, bioaccumulation and biomagnification, and organic carbon cycling, which could in turn cause substantial POP increases in water, soil, sediment, and biota. While these types of complex climate–POP interactions are not fully understood, they could be more problematic than climate sensitive outcomes leading to thermodynamic forcing and altered environmental partitioning (MacDonald et al., 2002).

Global warming will be expected to enhance partitioning of POPs and other pesticides to the atmosphere, though the increase in atmospheric concentrations of these pollutants may be offset by enhanced degradation (Bailey, 2004; Benitez et al., 2006; Dalla Valle et al., 2007; Sinkkonen and Paasivirta, 2000; Sweetman et al., 2005; Van den Berg et al., 1999; Wania and Mackay, 1996). Moreover, regions subject to increased storm intensity, frequency, and variability could experience pulses of chemical releases or runoff that might present acute risks to human health and wildlife populations (Bollmohr et al., 2007; Burgoa and Wauchope, 1995; Chiovarou and Siewicki, 2007; Dabrowski et al., 2002; Presley et al., 2006; Vu et al., 2006).

As for contamination at higher latitudes, some hypothesize that a reduction of the temperature gradient across latitudes could suppress the long-range transport of POPs (Beyer et al., 2003). Others present evidence that accelerated polar melting of snow, ice, and permafrost, as well as altered organic carbon cycling and metabolism, could remobilize and increase levels of archived pollutants and enhance their air to sea exchange (Blais et al., 2001; Macdonald et al., 2003; Magnuson et al., 1997; Meyer and Wania, 2008; Schindler et al., 1997). Climate change is also expected to result in the greater use of pesticides in regions experiencing increases in arable lands and

expansion of pest pressures (Chen and McCarl, 2001; Reilly et al., 2001, 2003). As a result, human and wildlife pesticide exposures and effects will shift. However, new, more efficacious pesticides and adaptive farming practices, such as altered plant varieties and planting regimens, could offset some of the expected increase in pesticide applications.

Increased temperature and salinity linked to climate change could enhance the toxicity of some POPs and other pesticides in aquatic biota. Altered biotransformation of contaminants to more bioactive metabolites appears to be an important mechanism by which climate change enhances chemical toxicity. Moreover, these climate change and contaminant interactions could compromise homeostasis and physiological responses, potentially impairing species fitness, reproduction, and development (Brian et al., 2008; Heugens et al., 2001; Schiedek et al., 2007).

The complex interactions between climate change and pollutants may be particularly problematic for species living at the edge of their physiological tolerance range. For most species, there are optimum ranges of temperature, salinity, pH, moisture, etc., and organisms living under conditions that approach their tolerance limits are often more vulnerable to additional stressors, such as climate change and chemical pollution (Gordon, 2003; Heath et al., 1994; Heugens et al., 2001; Patra et al., 2007). Species with narrow ranges of tolerance to changing environmental conditions may have difficulty acclimating to climate change. Pollutant exposures may further hinder the ability of organisms to acclimate and make them more susceptible to infectious and vector-borne disease. In addition, species with short generational times, such as microbes and insects, may adapt more successfully to climate change than those species with long generational times. Altered habitats caused by the rapidly changing climate also could trigger species migrations that ultimately push populations into sub-optimal regions where they may experience reduced overall fitness and diminished tolerance to toxicant exposures (Heugens et al., 2001; Schiedek et al., 2007).

Improving our understanding of the effects of multiple stressors on natural systems is an important challenge for environmental scientists. It has taken on more urgency as climate change is not only altering the fundamental structure and function of many ecosystems, but is impacting the distribution and toxicity of chemical pollutants. The vulnerability of human and wildlife populations to climate-sensitive chemical exposures, in the context of the many other stressors that are being altered with climate change, is the paramount question that requires more rigorous study. In addition, the effects of climate change on contaminant toxicity will almost certainly be non-linear, and an important question for future research will be to elucidate thresholds or tipping points in which contaminants as cofactors with other stressors lead to profound effects on ecosystems.

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SECTION D

Other Major Scientific Contributions

Pamela D. Noyes

1. **Thyroid Disruption Framework (Attached)**

My research background has focused on examining chemical effects and modes of action (MOAs) on the vertebrate thyroid system, development, and reproduction, and in the design and implementation of high throughput screening (HTS) assays to characterize chemical interactions with molecular targets. My research focus in both these areas has greatly informed my work in the OCSPP/OSCP as part of the Endocrine Disrupter Screening Program (EDSP). In particular, the EDSP is focused on screening and testing chemicals for their potential to disrupt the estrogen, androgen, and thyroid pathways, and has been incorporating HTS assays into its *in vivo* and lower throughput *in vitro* test battery. Though there have been important strides with the estrogen and androgen pathways, the thyroid pathway has lagged due to limitations in the availability of HTS assays to evaluate chemical effects on thyroid molecular targets as well as the biological complexity of chemical effects on the thyroid axis. More recently, however, it became clear to me that EPA/ORD progress with new *in vitro* HTS assays targeting the thyroid pathway has made it possible to start using these HTS approaches in chemical screening for thyroid bioactivity.

As a first step in this process I have been leading the development of EPA's framework to integrate HTS assays into the EDSP screening battery. This is a collaborative project that has involved working closely with over a dozen NCCT and NHEERL scientists to identify critical molecular targets for the thyroid pathway, of which there are many, and carry-out evaluation of the many different new and emerging thyroid HTS assays for their readiness to be used in hazard screening. This work has also involved determining how best to begin to integrate and link these mechanistic tools with animal-based studies, which are typically describing apical outcomes but not MOAs, thyroidal or otherwise. In addition to a great deal of work in thyroid toxicology, this work has required using my knowledge of adverse outcome pathway (AOP) approaches as we decided AOPs were a logical choice for organizing the differing types of assays collected across multiple levels of biological organization. I have been the lead coauthor of the framework document that has included communicating its approach, contents, and timeline to internal and external stakeholders. I have also worked closely with our HTS- and thyroid-focused research partners in ORD to complete the document.

The draft framework for peer review was completed under very fast timing (it was only just started this summer) and will now be evaluated as part of an SAP peer review later in the spring 2017. There is also the likelihood that this framework will be submitted for publication early next year. It has been rewarding to work with the smart and committed scientists that are participating on this effort. This framework will make an enormous difference in the Agency's progress on a technical complex mechanistic pathway. This type of approach for characterizing chemical effects on the thyroid axis has not been undertaken to date, and has major international implications for how other governments and organizations proceed with examining chemical thyroid disruption. It is poised to serve as a model both within EPA and in the international community for using HTS data to evaluate chemical effects on other toxicity pathways.

2. SETAC Pellston Workshop

I was invited to participate on a SETAC Pellston Workshop to examine the influence of global climate change on the scientific foundations and applications of environmental toxicology and chemistry (https://www.setac.org/news/111775/New-Paper-Series-on-Global-Climate-Change-Workshop-Now-Available.htm). These workshops, and the rigorous work that follows, are preeminent events that bring together leading scientists from academia, industry, and government from around the world. It was an honor to be able to work on such an important effort with global repercussions. This was the first international workshop to begin to incorporate toxicology considerations into discussions of global climate change as human health and ecological risk assessment are emerging as important tools in making decisions about how to adapt to climate change. In particular, we focused on proposing and designing a framework, using case study examples, to incorporate non-chemical parameters being altered by climate change (e.g., temperature, precipitation, etc.) into chemical risk assessment processes. I was asked to participate on the toxicity mechanisms workgroup based on my leadership and risk assessment background with EPA and the manuscript I spearheaded to examine the interactive effects of the changing climate on toxicity pathways, which was one of the first large scale scientific impact assessment of this problem (Noyes et al., 2009).

Under the Pellston, a series of papers were completed of which I worked with several collaborators in co-authoring the toxicology mechanisms framework (Hooper et al. 2013). While these papers were ultimately published, they represented a stand-alone compendium that together describe the best available science describing the interactive effects of climate change on contaminant exposures and effects, and importantly how to begin to assess and reduce these impacts. I was the lead co-author with Dr. Gary Ankley, ORD/NHEERL, in preparing the endocrine disruptor screening portions of the toxicity mechanisms framework, and was the primary author in preparing the section describing the potential effects of the changing climate on the toxicity of thyroid disrupting chemicals. I designed an AOP that integrated a great deal of mechanistic data for the thyroid axis pathway into a thyroid AOP network that described interactive effects from molecular initiating events (MIEs) through key biological events that culminate in adverse outcomes. I incorporated non-chemical parameters being altered by climate change into this thyroid AOP network as a tool for understanding chemical and non-chemical stressor interactions. This work represents one of the early thyroid AOP network constructs and was particularly important because it demonstrated how AOPs can serve as excellent tools for integrating non-chemical stressor data into pathway frameworks that could inform decision-making. It has been used as one of the models informing the Thyroid Disruption Framework described previously. I also researched and prepared the text describing the interactive effects of chemical exposures and increasing hypoxia predicted under climate change. The hypoxia pathway is an area that I did not have in-depth familiarity, but I was more than interested in tackling the literature to examine the potential interactive effects of dioxin and aromatic hydrocarbon exposures on the aryl hydrocarbon receptor (AhR) pathway in combination with cross-talk among hypoxia signaling pathways and teratogenicity. Finally, I presented outcomes of the Pellston workshop on behalf of our toxicity mechanisms subgroup at the 2011 SETAC meeting, and gave a seminar on this topic for the EPA NHEERL Mid-Continent Ecology Division in 2012.

3. UNEP/Norwegian EPA: Co-author on DecaBDE Risk Analysis

Decabromodiphenyl ether (DecaBDE) is an additive flame retardant primarily used in electrical and electronic equipment, as well as in textiles, where it is applied as a polymer back-coating to fabrics. Globally, DecaBDE has become the most used polybrominated diphenyl ether (PBDE) flame retardant. The United Nations Environment Programme (UNEP) has been considering whether to list DecaBDE as a Persistent Organic Pollutant (POP) under the Stockholm Convention. Norway was the key party proposing the listing and so was responsible for preparing the risk analysis describing the evidence for listing. The Norwegian EPA requested my assistance on a workgroup, along with other PBDE researchers in the field, to assemble the DecaBDE risk analysis (UNEP/POPS/POPRC.10/10/Add.2). In particular, Annex D to the Convention sets out criteria as to the required supporting evidence to determine a chemical's potential to be persistent, bioaccumulative, and toxic (i.e., PBT criteria), including the potential for long range transport. I was the co-lead author with my PhD advisor, Dr. Heather Stapleton, on the sections describing the evidence for the Environmental Fate (Section 2.2.) that included assessing the data and evidence for DecaBDE's: biological metabolism and debromination, bioavailability and tissue distributions, and bioaccumulation/biomagnification potential.

The extent to which DecaBDE bioaccumulates and is metabolized has been one of the more complex and controversial issues surrounding DecaBDE effects in humans and wildlife, in addition to its potential to be a thyroid disruptor that impairs development. Our review found that there is a high probability that DecaBDE is transformed in the environment and in biota to form substances, or act as precursors to lower polybrominated diphenyl ethers (PBDEs), which themselves are POPs. The scientific evidence supporting its metabolism and bioactivation to potentially more toxic, lower PBDE congeners has not always aligned. My more recent research in fish supports both bioaccumulation and reductive debromination of DecaBDE to lower PBDE congeners, but not oxidative metabolism, which is an important metabolic pathway in mammals. Other recent evidence also supports bioaccumulation and biotransformation. This more recent evidence of bioaccumulation and biotransformation appears to coincide with advances in analytical technologies and methods to detect DecaBDE and other higher PBDE congeners as historically they have been difficult analytes to measure in biota and environmental media. I also participated in the review of the hazard portion of the risk analysis, and was grateful that some of my laboratory research (Noyes et al. 2011, 2013) could be used in this evaluation.

In October 2015, the POP review committee adopted all the findings of the DecaBDE risk analysis. The POP review committee further recommended that DecaBDE be listed under Annex A to the Convention, which requires that manufacturers and end users take measures to eliminate the production and use of DecaBDE. Given that DecaBDE continues to be detected in humans, wildlife, and at high levels in the environment, it is a relief that this compound appears to be finally at a point where it will be listed alongside the other PBDE commercial mixtures as a POP.

Risk Profile on DecaBDE, United Nations Environment Program, POP RC Reports, UNEP/POPS/POPRC.10/10/Add.2: http://chm.pops.int/Default.aspx?tabid=2301

4. Special Issue on Climate Change and Toxicology

I was invited by the Chinese journal, Current Zoology to co-lead, with Dr. Sean Lema, California Polytechnic University, a special column to update the evidence on climate change and toxicology interactions (http://www.currentzoology.org/issuedetail.asp?volume=61&number=4&issue_id=552). This Special Column of papers on "Ecotoxicology in a Changing Global Climate" addresses and updates topical research and methods to advance our current understanding of climate change and toxicology interactions. We were particularly interested in capturing international efforts focused on endocrine disruption, and reached out to several leading researchers in the field to conduct research and reviews on several issues. These issues describe: climatic shifts in the Arctic Ocean that are leading to food web changes that are altering the dynamics of POP and mercury exposures (McKinney et al. 2015); interactions between pesticides, fertilizers, and rising temperatures (Di Lorenzo et al. 2015); climate change-toxicant interactions on estuarine biota (DeLorenzo et al. 2015); effects of elevated CO2 on trace metal exposures and effects (Ivanina and Sokolova 2015). Dr. Lema and I also published an updated peer review of our advancing understanding of how some classes of chemicals, particularly endocrine disruptors and metals, are influencing climate change sensitivities, and how climate change is affecting the adverse effects potential of other chemical classes (Noyes and Lema 2015). Together, these articles describe the state-of-the-art of how the toxicity and endocrine-disrupting effects of chemical pollution is being affected by the environmental disturbances associated with climate change. It has been my and Dr. Lema's hope that this special column serves as an essential update for researchers studying climate-chemical interactions, and to provide insights to continue research to elucidate and reduce the potential for amplified susceptibilities and tipping points that may lead to the reduced resilience or accelerated decline of species. The papers that were recruited and selected for inclusion are cited below.

DeLorenzo M, 2015. Impacts of climate change on the ecotoxicology of chemical contaminants in estuarine organisms. Curr Zool 61:641-652.

Di Lorenzo T, Di Marzio WD, Cifoni M, Fiasca B, Baratti M, Sáenz ME, Galassi DMP, 2015. Temperature effect on the sensitivity of the copepod Eucyclops serrulatus (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone. Curr Zool 61:629-640.

Ivanina AV, Sokolova IM, 2015. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. Curr Zool 61:653-668.

McKinney M, Pedro S, Dietz R, Sonne C, Fisk AT, Roy D, Jenssen BM, Letcher RJ, 2015. A review of ecological impacts of global climate change on persistent organic pollutant and mercury pathways and exposures in arctic marine ecosystems. Curr Zool 61: 617-628.

Noyes PD, Lema SC, 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife populations. Curr Zool 61:669-689.

5. Metals Framework

One of the major projects I worked on during my time as a coordinator on EPA's Risk Assessment Forum, which was positioned in NCEA at the time, involved extensive work as a technical panel member and coordinator to develop EPA's Framework for Metals Risk Assessment (https://www.epa.gov/risk/framework-metals-risk-assessment). The co-leads on this project were Dr. Anne Fairbrother, ORD/NHEERL (Retired) and Dr. Randy Wentsel, ORD/Office of Science Policy (Retired). My participation on the technical panel was extensive and involved drafting or assisting in the drafting of all aspects of the document, including particularly sections on assessing metals persistence, bioavailability and bioaccumulation potential. Another reason I am including this particular project is that, unlike Dr. Fairbrother and Dr. Wentsel, I am not a metals scientist. I immersed myself in the metals literature to understand the unique attributes of metals that make characterizing their environmental behavior and potential for biological effects much different than that of organic contaminants of which I have experience and training. I was responsible for managing the contract for technical white papers that informed the structure and content of the framework. This required distilling and synthesizing the data and results of these white papers for inclusion in the Framework. The Metals Risk Assessment Framework was a high profile project with substantial monetary impacts, and required that I work closely with our internal partners in EPA program offices, as well as our other government partners, especially those from the DOE, DOD, and OMB. This coordination with internal and external stakeholders required extensive negotiation of framework language. I managed the Risk Forums interactions with the SAB to carry out the peer review of the framework, and managed the Agency's response to public comments of which there were many. I also managed two major public meetings that were held to describe and invite input on science issues surrounding the framework.

6. Awards

I was awarded the 2016 Best Postdoctoral Publication award by the Society of Toxicology for my work to design and implement tools and approaches using embryonic zebrafish HTS assays of developmental toxicity to characterize the bioactivity potential of chemicals (Noyes et al. 2015). I was awarded the EPA STAR grant fellowship for my doctoral studies and my postdoctoral studies were funded under an NIH/NRSA fellowship. I have received awards for several presentations at international meetings, including the 2010 International Dioxin and 2013 Brominated Flame Retardant Meetings.

Framework for screening chemicals for thyroid bioactivity using *in vitro* high-throughput data and adverse outcome pathways

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Executive Summary

The US EPA's Endocrine Disruptor Screening Program (EDSP) was established to identify chemicals that may disrupt estrogen, androgen, and thyroid hormone signaling. The EPA has been implementing high-throughput screening (HTS) and computational methods to enhance screening efficiency and reduce cost and animal use in chemical testing. A conceptual framework is described that outlines how the EPA plans to use *in vitro* HTS data as part of a weight-of-evidence evaluations that includes animal-based assays to screen chemicals for potential thyroid activity. It relies on an Adverse Outcome Pathway (AOP) network as the principal organizing tool to: 1) link putative molecular-initiating events with *in vivo* effects observed in the current EDSP Tier 1 screening battery; 2) prioritize chemicals for EDSP Tier 1 screening; and 3) contribute to overall weight of evidence (WoE) evaluations. Chemicals may interact with the thyroid axis through many molecular initiating events (MIEs) that appear largely non-receptor mediated. Recent efforts to summarize thyroid-related MIEs and new HTS assays provide an opportunity to link *in vivo* data generated under the EDSP Tier 1 Screening battery to thyroid pathway MOAs. Thyroid AOP networks provide an ideal tool for defining causal linkages, strengths of evidence, and research needs across different MIEs targeted by HTS and the downstream sequence of intermediate events and apical outcomes measured by EDSP *in vivo* screening assays. Several *in vitro* HTS assays are now available and provide an opportunity to measure chemical effects on MIEs in the thyroid pathway. Ongoing advances in these tools and thyroid AOP networks provide the opportunity moving forward to characterize the linkages between new HTS and existing animal-based data that can be used to build predictive models using computational approaches to further advance chemical prioritization and WoE evaluations in support of risk assessment.

Introduction

The U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP) screens and tests chemicals for potential interference with estrogen, androgen, and thyroid hormone signaling to assess the risk of endocrine disruption in exposed humans and wildlife. To more quickly and cost-effectively screen chemicals for potential endocrine bioactivity, the EDSP has been integrating *in vitro* high-throughput screening (HTS) assays for bioactivity at key endocrine-related molecular targets (U.S.EPA 2015a). HTS assays and computational methods have been used to demonstrate the potential for a chemical to interact with estrogen and androgen receptors (Browne et al. 2015; Judson et al. 2015; U.S.EPA 2014a). In addition, *in vitro* HTS assays targeting thyroid disruption have recently become available or adapted for use in EDSP applications. Thyroid hormone signaling encompasses complex feedback loops involving the brain, thyroid gland, circulatory system, liver, and other target organs, collectively referred to here as the 'thyroid axis', that are critical to the development and physiological functioning of vertebrates (Crockford 2009; Dickhoff and Darling 1983; Heyland et al. 2004; Huang et al. 2015). Xenobiotics may interact with and perturb the thyroid axis through many molecular targets (as reviewed by Brucker-Davis, 1998; Capen and Marten, 1989; Crofton, 2008; Gilbert and Zoeller, 2010; Hurley et al., 1998; Murk et al., 2013; Zoeller and Crofton, 2000). The development and adaptation of *in vitro* assays to examine chemical interactions with molecular targets of thyroid disruption provide an opportunity to incorporate HTS technologies into chemical bioactivity screening efforts of the thyroid axis pathway.

This paper describes a conceptual framework (**Figure 4: AOP-informed Screening Framework**) for integrating and organizing *in vitro* HTS assays with *in vivo* animal studies to screen chemicals for their potential to perturb the thyroid axis. The conceptual framework is informed by Adverse Outcome Pathway (AOP) constructs as the principle organizing tool for the thyroid axis (Ankley et al. 2010). We describe an approach for integrating *in vitro* HTS assays with *in vivo* screening data to provide

mechanistic information to: 1) characterize the connections between *in vivo* effects observed in the current EDSP Tier 1 screening battery to a thyroid axis AOP; 2) inform chemical prioritization for EDSP screening; and 3) contribute to overall weight of evidence (WoE) determinations of a chemical's potential to disrupt thyroid axis pathways. The conceptual framework described here is designed with an eye toward EDSP longer term objectives of using mechanistic data derived from *in vitro* HTS assays as possible alternatives to EDSP screening assays as the availability and reliability of HTS data to predict *in vivo* outcomes for the thyroid axis is demonstrated (U.S.EPA 2014b).

A challenge of interpreting mechanistic data has been linking results measured at lower levels of biological organization (e.g., receptor, biochemical, cellular) to apical endpoints meaningful in risk assessment. To help address these challenges AOPs have been employed as organizing tools for integrating causal or correlative linkages among the biological events that lead to adverse outcomes (AOs) following chemical exposures (Ankley et al. 2010; Villeneuve et al. 2014). An AOP begins with a molecular initiating event (MIE) and culminates in an adverse outcome connected by a linear sequence of intermediate key events (KEs) measurable at increasingly complex levels of biological organization (**Figure 1**). AOPs aim to visualize and document the series of key events that lead to toxicological responses and are useful for assembling data derived from *in silico*, *in vitro*, and *in vivo* sources to evaluate the plausibility of events leading to an AO. For a particularly complex AO, it may be that multiple AOPs, each defined by a single MIE, may be required to fully appreciate the network of biology relevant to that toxicity. The EDSP is now using frameworks for the estrogen and androgen pathways that are informed by AOPs to: 1) integrate the diverse types of *in vitro* and *in vivo* data generated for screening chemicals; 2) assess the performance of HTS methods as alternatives to low-throughput *in vitro* data; and 3) evaluate computational models that use HTS data to predict downstream biological responses to chemicals (Browne et al., 2016, submitted).

The overall approach for screening and testing chemicals for their potential to interact with the thyroid axis is the same as that being implemented for the estrogen and androgen pathways in that the EPA is focused on evaluating important molecular targets and adverse outcomes with the best available data and tools, including using available and reliable HTS approaches, alongside or as an alternative to EDSP screening battery assays. This shared approach is intuitive to endocrine disruptor screening given that hormones share common biological features and lifecycles represented by stages of synthesis, transport, homeostatic regulation, receptor binding, and clearance that include molecular targets that may be susceptible to chemical interference (**Table 2**). Practical differences come into play for EDSP screening and testing based on our understanding of how chemicals may interact with these different molecular targets in a given endocrine pathway, and the availability of assays and predictive models to evaluate these chemical interactions.

With regard to the thyroid axis pathway, the EDSP screening battery currently lacks *in vitro* assays relevant to chemical perturbation of their associated molecular targets, and as a consequence, integration of related HTS data into the EDSP screening battery is less straightforward than for the steroid hormone pathways where such *in vitro* assays are already part of the screening battery. Nonetheless, like with the estrogen and androgen pathways, characterization of the linkages between thyroid-related molecular targets and *in vivo* outcomes is critical in order to accurately understand the manifestation of thyroid disruption. For the steroid hormones, EDSP has emphasized chemical interactions with nuclear receptors because they have been shown to be prominent targets, albeit not the only targets, for environmental chemicals (Arnold et al. 1996; Blair et al. 2000; Kavlock et al. 1996; Kuiper et al. 1998). A variety of available and reliable HTS assays have been available for many years to measure chemical interactions with steroid hormone receptor activity, and the Agency is now using computational toxicology tools to screen chemicals for interaction with ERs (Browne et al. 2015; Judson et al. 2015) and is adopting similar approaches for the AR signaling pathway (U.S.EPA 2014a). In

contrast, strong evidence supports that chemicals can potentially perturb the thyroid axis through many molecular initiating events (MIEs), which represents the initial point of chemical interaction at a molecular level that starts the AOP (**Table 3**). In addition, unlike the steroid hormones, xenobiotics are probably not interacting predominantly with the thyroid hormone receptor (Murk et al. 2013; OECD 2014). Given the number of MIEs demonstrated or hypothesized to be involved in chemical-mediated disruption of the thyroid system, the use of computational toxicology tools for the thyroid pathway will require a broader approach from evaluation of the estrogen and androgen pathways. Efforts to develop and adapt *in vitro,* thyroid-related screening technologies for toxicology applications are making progress, and an AOP-informed screening framework in **Figure 4** provides a process for integrating these HTS data into the EDSP screening battery. EPA is in a position to begin considering current HTS assays for use in prioritization for Tier 1 screening, and as more HTS assays covering more molecular targets become available, the Agency will be in a position to use these assays to inform WoE evaluations and as alternatives to in vivo animal studies in the Tier 1 battery.

Background on U.S. EPA's EDSP

The EPA developed the EDSP in response to statutory mandate in the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act (FQPA) of 1996, to develop a screening program to evaluate chemicals for estrogenicity and provided authority to examine other endocrine pathways (FFDCA 1996; FQPA 1996). Congress also amended the Safe Drinking Water Act (SDWA) in 1996 and provided EPA with authority to evaluate chemicals found in sources of drinking water to which substantial populations may be exposed (SDWA 1996). In response, the Agency convened the Endocrine Disruption Screening and Testing Advisory Committee (EDSTAC), composed of experts from academia, industry, and government, to provide advice on EDSP design and implementation. The EDSTAC recommended that the EDSP evaluate chemical effects on the androgen and thyroid pathways, in addition to estrogenic effects, in wildlife and humans (EDSTAC 1998). In

addition, the EDSTAC recommended a two-tiered screening and testing strategy to link mechanistic data to apical endpoints and conceptually organized the testing into 'estrogenic', 'anti-estrogenic', 'androgenic', 'anti-androgenic', and 'thyroid-active' endocrine pathways (**Table 1**). Tier 1 was designed to screen for potential chemical interactions with the estrogen, androgen, and thyroid pathways, and Tier 2 testing was intended to characterize chemical effects identified as potentially active in Tier 1 screening. The battery of five *in vitro* and six short-term *in vivo* Tier 1 screening assays was intended to be considered collectively to maximize sensitivity while reducing the impact of limitations of any one assay. A WoE assessment including the results of the Tier 1 battery, along with any other scientifically relevant information (OSRI), forms the basis for whether subsequent Tier 2 testing is necessary (U.S.EPA 2011). There are four comprehensive, *in vivo* Tier 2 tests that include multiple vertebrate taxa (mammals, fish, birds, amphibians) intended to establish dose-response relationships and characterize adverse outcomes for risk assessment.

At present, roughly 10,000 compounds are subject to EDSP statutory mandate. The universe of chemicals includes data-rich chemicals, like pesticide active ingredients that require toxicity testing prior to registration, as well as chemicals known or anticipated to occur in drinking water, such as disinfection byproducts, pharmaceuticals, and industrial feedstocks, which may not have complete toxicological profiles (U.S.EPA 2012). Initial test orders were issued in 2009 for EDSP Tier 1 screening on 67 chemicals; 58 pesticide active and nine pesticide inert ingredients (U.S.EPA 2009). Fifteen of these chemicals were cancelled or discontinued by the pesticide registrant and are no longer in use, and WoE evaluations were completed for the remaining 52 chemicals (U.S.EPA 2015b). Of the 52 chemicals evaluated, 18 showed potential interaction with the thyroid axis, and EPA recommended a long-term amphibian growth and development assay (LAGDA; OCSPP 890.2300) and/or special thyroid assay be conducted for 7 of the 18 pesticides. The special thyroid assay in rats is a non-guideline study that has been required by EPA's Office of Pesticide Programs (OPP) in lieu of the rat developmental neurotoxicity study to

generate specific thyroid-related data in pregnant and nursing dams, their fetuses and newborns (U.S.EPA 2005). The proportion of chemicals that were potentially bioactive in the EDSP Tier 1 screening battery along with the large number of chemicals subject to the EDSP underscores the critical need for methods that can be employed for more rapid prioritization and hazard characterization.

Chemical Screening and Testing for Thyroid Axis Bioactivity

Of the 11 assays in the EDSP Tier 1 screening battery, three *in vivo* assays include thyroid-relevant endpoints: Male Rat Pubertal Assay (OCSPP 890.1500), Female Rat Pubertal Assay (OSCPP 890.1450), and the Amphibian Metamorphosis Assay (AMA; OCSPP 890.1100). The *in vivo* pubertal assays in male and female rats measure chemical effects on the reproductive and thyroid systems of neuroendocrine-intact, peripubertal animals. Thyroid-specific endpoints measured in the pubertal assays include serum concentrations of thyroxine (T4) and thyroid stimulating hormone (TSH), as well as thyroid weight and histopathology. The AMA targets chemical effects on the vertebrate thyroid as Amphibian metamorphosis is dependent on well-described and observable events that are TH mediated (Denver 1998; Fort et al. 2007), which is the biological principle supporting use of the AMA in screening for thyroid disruption. Endpoints measured in the AMA include thyroid histopathology, timing of metamorphic transitioning, and other thyroid-modulated developmental parameters (hind limb length, snout-vent length).

The EDSP screening and testing strategy was intended to link mechanistic data to adverse outcomes. Though known thyroid toxicants were included during development of the EDSP Tier 1 screening battery, at the time, research and development of the set of *in vitro* assays relevant to screening for thyroid axis bioactivity was less mature. A number of recent efforts have reviewed the state of the science and research tools for examining chemical-thyroid system interactions. These reviews serve as an important foundation and catalyst for the EDSP-specific framework outlined here. Recently, the U.S. EPA, National Institutes of Health (NIH), World Health Organization (WHO),

Organisation for Economic Co-operation and Development (OECD), and academic organizations have been developing tools to characterize the underlying AOPs by which chemicals may perturb the thyroid pathway leading to potential adverse outcomes. For example, a 2011 workshop was sponsored by the Assuring Safety without Animal Testing (ASAT) Foundation to review the MIEs and AOPs for chemically induced thyroid disruption and assess the current availability and reliability of different *in vitro* tools (Murk et al. 2013). Similarly, the OECD produced a scoping document that reviewed the performance and adequacy of existing *in vitro* thyroid-related assays, and identified others that could serve to fill data gaps with further optimization (OECD 2014).

Use of HTS and Computational Tools by the EDSP

The EDSTAC report to EPA in 1998 recommended inclusion of computational toxicology approaches in endocrine bioactivity screens, including quantitative structure activity relationships and HTS, although at that time the availability of relevant tools and methods was relatively limited (EDSTAC 1998). This focus by the EDSTAC was consistent with subsequent conclusions of the National Research Council (NRC) in their report on *Toxicity Testing in the 21st Century* that indicated that a broad transformational shift in toxicity testing was needed from whole animal test systems to *in vitro* technologies and bioinformatics (NRC 2007). The NRC report emphasized advances in the availability and reliability of *in vitro* and *in silico* HTS tools for toxicity screening and promoted a shift towards computational approaches to predict chemical bioactivity, increase the rate of screening, and reduce animal testing. In response to the NRC report, the EPA, National Institute of Health (NIH), and Food and Drug Administration (FDA) formed the Tox21 consortium (http://www.epa.gov/ncct/Tox21/) to apply HTS technologies to screen thousands of chemicals for toxic effects (Tice et al. 2013). In addition, EPA launched its ToxCast™ program in 2007 (http://www2.epa.gov/chemical-research/toxicity-forecasting) to further develop *in vitro* HTS platforms for testing large environmental chemical libraries (Dix et al. 2007; Judson et al. 2010; Kavlock et al. 2008). With these advancements, the EDSP has been undergoing

a multi-year transition under its EDSP21 initiative to use HTS technologies and computational methods (U.S.EPA 2014b, 2015a).

Inclusion of HTS data in thyroid bioactivity screening is advantageous because although the pubertal and AMA Tier 1 assays are intended to screen for thyroid-active chemicals, altered time to development or other *in vivo* endpoints may be influenced by a variety of biological processes, and HTS tools may indicate a putative MIE and AOP relevant for endocrine bioactivity. As portrayed in **Figure 4**, *in vitro* HTS data can be readily integrated with Tier 1 assays in WoE evaluations of a chemical's potential to interact with the thyroid pathway, and we present a framework for organizing these diverse data in the subsequent section on application of thyroid-related AOPs to the EDSP. The incorporation of *in vitro* assays and mechanistic screening information for the thyroid axis pathway enhances the EDSP process in four major ways: (1) identifying potential thyroid-related AOPs of interest for the xenobiotic in question; (2) providing a rapid means of prioritizing chemicals for further *in vitro* or *in vivo* screening and testing for thyroid-related endpoints under the Tier 1 battery; (3) identifying molecular targets that are conserved across taxa to evaluate for cross-species relevance; and 4) aiding interpretation of potentially thyroid-related adverse outcomes observed in Tier 2 tests.

MIEs and High-Throughput Methods Targeting the Thyroid Pathway

Recent reviews of thyroid disruption have summarized thyroid pathway MIEs that are demonstrated or hypothesized targets of environmentally-relevant chemicals and reviewed the status of HTS assays for these MIEs (Murk et al., 2013; OECD 2014). Table 3 summarizes the status of HTS technologies targeting these MIEs as: 1) Existing – assays are either currently in use or capable of HTS; 2) Promising – assays are currently in use that have the potential to be adopted or further optimized for HTS; or 3) Research and development (R&D) – no HTS assays exist for the MIE or existing assays will require basic R&D prior to use. The supplementary materials provide a detailed summary of the underlying data supporting the toxicological relevance of each MIE and the status of different HTS

technologies. HTS methods will continue to develop as more assays become available and current assays are refined and adapted for HTS applications. Currently, *in vitro* HTS assays or lower/medium throughput *in vitro* assays potentially amenable to high-throughput platforms and capable of measuring chemical interactions with MIEs on the thyroid pathway include those targeting:

- TH biosynthesis – Sodium-iodide symporter (NIS), Thyroperoxidase (TPO);
- Serum TH-binding protein – Transthyretin (TTR), Thyroid binding globulin (TBG);
- TH peripheral tissue metabolism – Type 1 iodothyronine deiodinase (D1) and markers of nuclear receptor activation and metabolism (e.g., constitutive androstane receptor (CAR), pregnane X receptor (PXR), uridinediphosphate glucuronosyl transferases (UDPGTs));
- TH transmembrane transport – Monocarboxylate transporter 8 and 10 (MCT8, MCT10), Organic anion transporter polypeptide 1c1 (OATP1C1); and
- Receptor-ligand binding – TR transactivation (TRTA), TSH receptor (TSHr), Thyrotropin releasing hormone receptor (TRHr)

Below we highlight the status of HTS assays and discuss the relevance of several of the MIEs as potential targets of chemicals in the context of more well-studied contaminants (e.g., polychlorinated biphenyls (PCBs), organochlorine pesticides), halogenated flame retardants, organophosphate pesticides, and other industrial and consumer product chemicals (**Figure 2**).

TH Biosynthesis: A number of chemicals have been shown to disrupt the thyroid system by impairing the thyroid gland's production of TH (**Figure 2, inset**). The most well characterized MIEs in the thyroid pathway involve xenobiotic inhibition of iodine uptake into the thyroid gland (NIS inhibition) and interference with hormone synthesis in the gland by inhibition of TPO activity. The most well characterized MIEs in the thyroid pathway involve chemical inhibition of NIS and/or TPO bioactivity. Perchlorate, which is used as a propellant in rocket fuels and in other industrial applications (Trumpolt et al. 2005), is an example of a model inhibitor of NIS-mediated transport of iodide from the blood into

thyroid follicular cells (Clewell et al. 2004; Dohan et al. 2007; Greer et al. 2002; Tietge et al. 2005). As iodine is essential for production of TH, its diminished supply causes decreases in serum T4, with elevated TSH, and thyroid gland hypertrophy with adult exposures in the EDSP Tier 1 male pubertal assay (Stoker et al. 2006). Developmental exposure to perchlorate also reduces serum T4 in pregnant and nursing dams and impairs synaptic transmission in the brains of offspring (Gilbert and Sui 2008). Similarly, depleted serum T4, thyroid gland pathology and delayed metamorphosis occur in Amphibians following exposure to perchlorate (Tietge et al. 2005; Tietge et al. 2010), emphasizing the conservation of NIS across vertebrate taxa. An in vitro radioactive iodide (^{125}I-) uptake (RAIU) assay coupled to a human NIS-expressing HEK293T cell line has been developed and adapted for use as an HTS assay to identify chemicals that may inhibit ^{125}I- uptake by NIS (Hallinger et al. 2016; Murr et al. 2016). In addition, several other existing medium-throughput assays are available to assess iodide uptake and are potentially amenable to further HTS development (Cianchetta et al. 2010; Lecat-Guillet et al. 2008; Rhoden et al. 2008; Waltz et al. 2010).

Chemicals may also perturb TH biosynthesis by inhibiting TPO. Methimazole and 6-propyl-2-thiouracil (PTU) are pharmaceuticals used to treat hyperthyroidism in humans and animals and as model goitrogens in basic research (Cooper et al. 1984; Nakashima et al. 1978; Taurog 1976; Zoeller and Crofton 2005). A proposed AOP for PTU toxicity in developing humans includes maternal TH insufficiency related to TPO inhibition along with peripheral inhibition of D1 (Leonard and Rosenberg 1978), leading to reduced TH levels in the fetal brain, altered TH-mediated gene expression, structural brain deformities, and abnormal behavioral outcomes depending on the timing of administration (Zoeller and Crofton 2005). TPO is a relevant target for disruption across taxa, as TPO inhibitors reduce thyroid hormone synthesis in amphibians and avian species (Coady et al. 2010; Grommen et al. 2011; Hornung et al. 2015; Rosebrough et al. 2006; Tietge et al. 2010; Tietge et al. 2013). The peroxidase catalytic domain of TPO is also highly conserved between rats, pigs, and humans (Paul et al. 2013). Thus, TPO

presents a relevant, cross-species target for the EDSP. A fluorogenic HTS assay using Amplex UltraRed™ to detect TPO inhibition (AUR-TPO) has been developed and used to screen the ToxCast Phase 1 and 2 chemical libraries (~1000 chemicals; Paul-Friedman et al., 2016).

Serum TH-binding proteins: Some classes of environmental chemicals, particularly halogenated aromatic chemicals, have been shown to be ligands that can bind to the serum TH transporter proteins, notably TTR, thereby displacing native TH, which is hypothesized to increase TH clearance leading to reduced serum TH concentrations. For example, in vitro evidence demonstrates that some brominated flame retardants and their hydroxylated metabolites (Hamers et al. 2006; Marchesini et al. 2008; Meerts et al. 2000; Ren and Guo 2012), polychlorinated biphenyls (PCBs) (Cheek et al. 1999; Gutleb et al. 2010; Ucan-Marin et al. 2010), and perfluorinated compounds (PFCs) (Weiss et al. 2009) may competitively bind TTR (and to a lesser extent TBG) and displace T4. A surface plasmon resonance (SPR)-based biosensor assay for TTR and TBG has been developed that provides medium to high-throughput testing capabilities with commercially available technologies (Marchesini et al. 2006; Marchesini et al. 2008).

TH peripheral tissue metabolism: In addition to inhibiting TH binding to serum transport proteins, organohalogen chemicals have been shown to perturb TH homeostasis by altering the expression and activity of Phase 2 conjugating enzymes, uridine diphosphate glucuronosyltransferases (UDPGTs) and sulfotransferases (SULTs), thereby increasing TH catabolism, and in some cases reducing serum TH levels by apparent excretion (Barter and Klaassen 1994; Butt et al. 2011; Hood et al. 2003; Palace et al. 2008; Szabo et al. 2009; Yu et al. 2009; Zhou et al. 2002). ToxCast HTS assays are available to assess a chemical's ability to bind and activate specific hepatic nuclear receptors (e.g., CAR; PXR), but these assays may not be as selective for identification of putative thyroid-disrupting chemicals. *In vitro* HTS assays that target a chemical's ability to measure induction of Phase 2 enzymes known to catabolize thyroid hormones are still in development. The iodothyronine deiodinase (Dio) enzymes (D1, D2, D3) are critical to the spatial and temporal maintenance of TH homeostasis and targets for chemical disruption

306 across vertebrate taxa (Murk et al. 2013). Recent *in vitro* and *in vivo* evidence has shown that some
307 organohalogens can alter the expression and activity of Dio enzymes in human liver microsomes (Butt et
308 al. 2011), rodents (Hood and Klaassen 2000; Szabo et al. 2009), fish (Dong et al. 2013; Noyes et al. 2011;
309 Noyes et al. 2013; Picard-Aitken et al. 2007) and birds (Farhat et al. 2013). Renko and coworkers have
310 developed lower throughput colorimetric assays that allow measurement of chemical effects on
311 deiodination activity as catalyzed by D1, D2, and D3 (Renko et al. 2012; Renko et al. 2015) as well as by
312 iodotyrosine deiodinases (IYD)(Renko et al. 2016). These assays are being adapted by EPA in designing
313 HTS formats to measure chemical effects on Dio enzyme activity with an HTS assays targeting D1
314 interference near completion.

315 **TH transmembrane transport:** MCT8, MCT10, and OATP1C1 have been shown to be specific and
316 potent TH transporters across plasma membranes (Friesema et al. 2003; Pizzagalli et al. 2002; van der
317 Deure et al. 2008; Visser et al. 2008), with mutations in the human MCT8 gene producing
318 hypothyroidism and severe neurological deficits (Friesema et al. 2004; Heuer et al. 2005). Limited in vivo
319 evidence suggests that some PBDE flame retardant chemicals may alter the expression of genes
320 encoding MCT8 and OATP1C1 (Noyes et al. 2013; Richardson et al. 2008).

321 **Receptor-Ligand Activity:** Limited and in some cases divergent data suggest that some
322 chemicals, including some brominated flame retardants such as the PBDEs (Kitamura et al. 2008; Lema
323 et al. 2008; Noyes et al. 2013; Ren et al. 2013) and tetrabromobisphenol A (TBBPA) (Kitamura et al.
324 2005), perfluorinated compounds (Ren et al. 2015), and other chemicals used in plastics (e.g. bisphenol
325 A (BPA)) (Moriyama et al. 2002), can alter the expression and activity of TRs. However, ToxCast screens
326 of large chemical libraries using transactivation assays targeting TRα and TRβ have revealed few positive
327 results, suggesting that binding may be restricted to a limited set of structures
328 (https://actor.epa.gov/dashboard/). Similarly, HTS assays measuring chemical binding to TRHr and TSHr
329 are not predicted to be important MIEs for xenobiotic perturbation, although this is an area where

research is limited. To empirically evaluate the environmental relevance of these targets, HTS assays for TRHr and TSHr are currently being developed and analyzed as part of ongoing ToxCast/Tox21 efforts.

AOP Networks as Organizing Frameworks for Thyroid Bioactivity Screening

To provide a more realistic representation of biological complexity, multiple AOPs can be integrated to construct AOP networks that include multiple MIEs that share at least one KE leading to one or more adverse outcomes (Villeneuve et al. 2014). AOP networks are particularly useful for evaluating thyroid MIEs that are potential targets for environmental chemicals. A detailed AOP network can be used to organize and evaluate thyroid data in a research context and can be used to develop new assays, examine the evidence for causality between KEs in the AOP, and develop quantitative relationships between KEs (**Figure 3**). A less detailed, higher level screening framework (**Figure 4**) that is informed by the more detailed thyroid AOP network and restricted to endpoints that are available for regulatory decision-making can be useful for integrating HTS data, and other KEs or biomarkers, with endpoints included in the EDSP Tier 1 screening battery.

Many of the MIEs identified in recent reviews (Murk et al. 2013; OECD, 2014) can be mapped to an AOP network for the thyroid axis that includes KEs and KE relationships that may culminate in apical responses in human health and wildlife models (**Figure 3**). Currently available HTS assays to measure chemical-induced MIEs are highlighted in purple, and endpoints collected in EDSP Tier 1 screening assays are highlighted in red. The purpose of this AOP network is not to recapitulate more formalized thyroid AOPs (e.g., those captured on the AOP-Wiki). Rather, Figure 3 is intended to illustrate how EPA is organizing data into a logical and cohesive framework that links mechanistic data to adverse outcomes needed for regulatory decision-making. Thus, an adverse outcome (e.g., AMA: altered hind limb length (HLL), snout-vent length (SVL), time to metamorphosis) may be the result of interactions with many MIEs, and conversely, a single MIE may be linked to many adverse outcomes that occur in specific vertebrate models and life-stages. Describing KE relationships between MIEs and adverse outcomes can

be helpful in clarifying data gaps and identifying research needs for regulatory decision-making. For example, there are a number of MIEs not highlighted in purple for which HTS assays are not currently available even though in some of these cases lower throughput in vitro assays have been developed (e.g., iodotyrosine deiodinase (IYD) interactions). Unlike more formalized AOPs that seek to characterize the strength of evidence, the linkages connecting MIEs, KEs, and AOs in Figures 3 and 4 may be considered hypothesized, biologically plausible relationships that vary depending on the strength of the empirical data. Similarly, there are potential adverse outcomes that may be captured in the EDSP Tier 1 screening battery (AMA endpoint) but other effects reported in the scientific literature not captured by EDSP assays that may provide data relevant for decision-making.

Thyroid AOP networks for the thyroid pathway also can be used to clarify MIEs and KEs that are common to multiple taxa but that may lead to different adverse outcomes depending on the animal. For example, inhibition of D1 may lead to reduced levels of serum and tissue TH that may alter downstream genomic activity leading to consequent AOs that vary by test model (e.g., mammals = altered neurodevelopment; amphibian = impaired metamorphosis; fish = reduced survival). These AOs may be captured as part of the EDSP screening battery or OSRI. Similarly, thyroid AOP networks can be used to shows points where several MIEs converge at a shared KE. These shared KEs may be particularly useful in identifying biomarkers that could be used in designing screening assays and building computational models. For example, several of the MIEs may induce a cascade of KEs that proceed through decreased concentrations of TH in serum and target tissues, thereby providing a potentially relevant biomarker for use in research design, chemical screening, and predictive modeling. The male and female pubertal assays include circulating thyroxine (T4) and thyroid-stimulating hormone as endpoints to indicate potential effects on thyroid status. Serum and tissue T4 concentrations are key biomarkers that can be evaluated in the design of additional studies to fill data gaps or further clarify chemical effects on poorly understood thyroid pathways (DeVito et al. 1999).

Though informative for identifying research needs and understanding causal linkages, the thyroid AOP network in Figure 3 includes key events and key event relationships for which data are not currently available for a WoE evaluation of a chemical's potential interaction with the thyroid axis. However, it may be possible to develop WoE evaluations that suggest a possible endocrine-specific mechanism for an observed *in vivo* finding based on data from *in vitro* HTS assays that may indicate perturbation of a thyroid-related MIE, along with EDSP Tier 1 assay information that demonstrate apical effects that may be thyroid-related (Figure 4). The reduced thyroid AOP network in Figure 4 is limited to data that may be available for evaluating thyroid bioactivity using these two data types, HTS assays and EDSP Tier 1 assays, in a regulatory context. In addition, all available data including information from published literature can be similarly evaluated *prior* to WoE evaluations. Though the putative AOP network illustrated in Figure 4 is highly reductive and an abbreviated representation of many complex biological processes, it is nonetheless useful for organizing and integrating diverse data available in a regulatory context.

Regulatory data gaps and uncertainties (Figure 4) can also be captured in this type of putative AOP network where research suggests MIE or KE may be important but assays are not currently available for that endpoint. For example, chemical mediated increases in TH catabolism and clearance are an important MOA of thyroid perturbation (Figure 4; Table 3; supplement). ToxCast HTS assays target some MIEs that may provide indirect links (e.g., hepatic PI nuclear receptor activation); however, HTS assays do not measure chemical interactions with this MIE directly and *in vivo* Tier 1 assays do not evaluate these endpoints (beyond changes in liver weight). With continuing advances in the availability and reliability of HTS methods, it will be possible to expand the putative AOP network for thyroid bioactivity to allow for interrogation of additional molecular targets and apical responses, and to further clarify data gaps as data describing the biological convergence of KEs advances.

Another useful tool of thyroid AOP networks (Figures 3 and 4) relates to characterizing species sensitivities to inform chemical screening particularly when evaluating the extent to which data from one animal model can be used to predict potential adverse outcomes in another. Based on the conserved structural and functional features of the vertebrate thyroid system, the EDSP Tier 1 AMA was intended to be a sensitive screen for chemical effects on the vertebrate thyroid system, rather than an organism-specific ecotoxicity test. Though thyroid histopathology in the AMA has been found to be a reliable endpoint to detect chemical mediated reductions in serum TH, gross developmental changes (e.g., hind limb length) have been found to be less sensitive to hypothyroidism likely due to compensatory feedbacks and tissue plasticity (Pickford 2010). In addition to toxicokinetic and toxicodynamic differences in chemical metabolism, there are differences in thyroid physiology between mammals and amphibians so it is possible that effects between the AMA and rodent pubertal assays under the Tier 1 screening battery may not always align. These physiological differences include, but are not limited to: differences in thyroid hormone stability and kinetics due to differences in predominant binding proteins; differences in thyroid hormone storage capacity in follicular cells; differences in nuclear receptor isoforms expressed across species (Paul et al. 2013; Omiecinksi et al 2011); and differences in the predominant mechanisms of thyroid hormone clearance. These differences in thyroid physiology may manifest in different responses in the AMA and pubertal assays. A systematic review to identify thyroid reference chemicals for EDSP Tier 1 and Tier 1-like assays found that of 35 candidate compounds identified and tested in all three thyroid-related EDSP Tier 1 assays (Male/Female Pubertals, AMA), about half (19 chemicals) were found to be 'positive' for thyroid pathway effects in just one of three assays with differences in potency across vertebrate model and sex (Wegner et al. 2016). A clear difficulty in constructing a WoE is understanding what might be relevant across species, and the incorporation of HTS data on MIE perturbation may provide a means of evaluating species differences in thyroid-related outcomes by enabling an assessment of the relevance of chemical perturbation of these MIEs, which may demonstrate species-specific expression or activity profiles, across different species.

This type of cross-species assessment, focusing on the MIE and early KEs, is commonly performed in support of MOA-based human health risk assessment for cancer and non-cancer MOAs (Rouquie et al., 2014; Tinwell et al., 2014; Papineni et al., 2015; Rasoulpour et al., 2015; Ellis-Hutchings et al, 2014).

Differences in the expression and activity of deiodinases across animal models, which represent MIEs within the AOP network, provide an example of how differences in thyroid physiology might be relevant to chemical mediated thyroid perturbations. D2 is expressed abundantly in the developing brains of both mammals (Croteau et al. 1996) and amphibians (Dubois et al. 2006), and studies suggest its role in maintaining TH homeostasis in brain neurons of developing rodents (Galton et al. 2007; Guadano-Ferraz et al. 1999) and in controlling amphibian metamorphosis (Becker et al. 1997; Cai and Brown 2004). In contrast, however, the importance of D2 in maintaining TH homeostasis and in consequent physiological actions in the developing fish brain is much less clear (Frith and Eales 1996; Johnson and Lema 2011; Noyes et al. 2013). Similarly, D1 has been localized to the livers of mammals and fish but data suggest that hepatic T4 deiodination to T3 is catalyzed largely by D1 in mammals and D2 in fishes (Gereben et al. 2008; Kohrle 2000; Mol et al. 1993; Orozco and Valverde-R 2005). Finally, the model goitrogen PTU has been shown to inhibit D1 in mammals and birds but appears to have less specificity in inhibiting D1 in amphibians and fish (Galton 2005; Kohrle 1999). Thus, chemical interactions with deiodinases could lead to a cascade of differential events depending on the species (and lifestage), or alternatively might produce different adverse outcomes but with a shared MOA. Indeed, it is unlikely a chemical will elicit the same response across multiple taxa, sexes, and life stages, except perhaps the most potently thyroid-active chemicals (Wegner et al. 2016). AOP networks provide a construct for capturing these types of potential species sensitivities by linking HTS data and other mechanistic KEs to model-specific adverse outcomes. This type of information can in turn inform when screens for thyroid bioactivity could benefit from combining lines of evidence across multiple species or alternatively when species-specific data should be separated for regulatory decision-making.

To screen chemicals for interactions with the ER, EPA has developed a ToxCast ER model that integrates results from 18 high-throughput *in vitro* assays that provide information at a number of points in the ER signaling pathway and rely on a variety of assay technologies (Judson et al. 2015). The redundancy and diversity of HTS technologies provides a great deal of confidence in "positive" and "negative" chemical interactions and allows discrimination of false positive signals. The ToxCast ER model was evaluated as a potential alternative for the three Tier 1 assays targeting chemical interactions with the ER (i.e., ER Binding/OCSPP 890.1250; ER Transcriptional Activation (ERTA)/OCSPP 890.1300); and Uterotrophic Assay/OCSPP 890.1600), and predicted the activity for a structurally diverse set of reference chemicals with an overall accuracy of 93% and a false negative rate of 7% (Browne et al. 2015; U.S.EPA 2015a). Based on this predictive performance, the Agency is now accepting ToxCast ER model data as an alternative to the ER-targeted assays in the Tier 1 battery. A similar computational model based on 11 AR HTS assays has been developed and the balanced accuracy of this model against agonist and antagonist reference chemicals is >95% (Kleinstreuer et al., 2016).

Though *in vitro* HTS assays are available for several MIEs in the thyroid pathway, there are not multiple assays for most MIEs (as is the case for ER and AR), and there are not existing Tier 1 *in vitro* assays to replace with these HTS alternatives. For example, in addition to the HTS assay for TPO inhibition a medium-throughput orthogonal GUA oxidation assay is also available (Chang and Doerge 2000; Paul-Friedman et al. 2016; Paul et al. 2013). Thus, for an MIE like TPO inhibition, two screening assays with medium to high throughput capacities are available. However, for most MIEs in the thyroid pathway multiple *in vitro* assays are not available (Murk et al., 2013; OECD 2014). Moving ahead, thyroid-related HTS assay data may be helpful not only for prediction of possible effects, but also for prioritization applications, despite some uncertainty regarding the potential for a chemical to elicit activity at a particular MIE target. Development of confirmatory, more biologically complex assays that link MIEs with early KEs may increase the confidence in results and the utility of *in vitro* assays for

prioritizing chemicals for additional thyroid activity screening. Herein the HTS assays that are relevant to EDSP Tier 1 assays with measures of thyroid disruption clearly enables the use of these HTS data in WoE assessments.*in vitroin vivoin vivo*

Looking Forward – Building Predictive Models

AOP networks for the thyroid pathway (Figures 3 and 4) provide the organizational context for integrating new technologies into the EDSP Tier 1 screening battery, and provide a flexible construct for integrating additional *in vitro* HTS technologies as they become available. With these promising advances, there is a great deal of interest in building predictive models using computational toxicology methods as a more efficient means of prioritizing the universe of chemicals for testing under the Tier 1 battery and in carrying out WoE evaluations of chemicals for testing under EDSP Tier 2.

AOP networks for the thyroid pathway can be used in building predictive models by defining the causal and/or correlative linkages from *in vitro* HTS data to KEs and/or adverse outcomes measured in animal testing. Information from AOPs can be used to develop a fit-for-purpose approach for characterization of the performance of *in vitro* HTS assays or models that integrate these assays to predict these downstream KEs or adverse outcomes. Models that combine information from assays that measure potential effects on different MIEs will be useful in creating a prioritization scheme for thyroid pathway bioactivity.

Initial computational modeling approaches could integrate HTS results for screening at multiple MIEs in order to generate a priority score or prediction regarding the likelihood that a chemical may be capable of perturbing the thyroid pathway. Developing models that predict adverse outcomes from HTS data for MIEs will be more challenging due to gaps in complete understanding of all of the quantitative key event relationships (Becker et al., 2015 Reg Tox Pharm) within the thyroid AOP network. Though qualitative evidence is generally strong that the MIEs listed in Table 3 relate to thyroid hormone

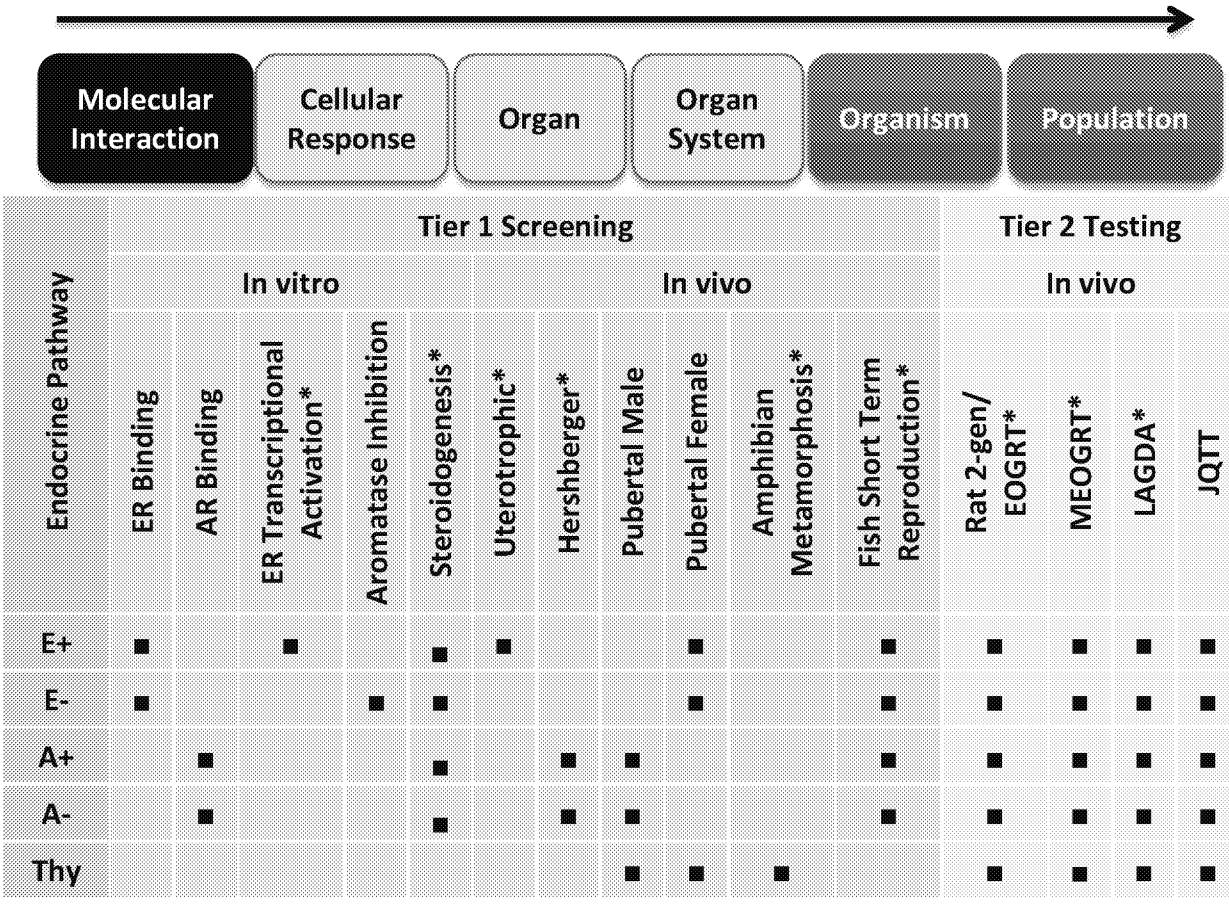
perturbation, quantitative understanding of how changes in some of the MIEs, particularly as indicated by HTS assay results, relate to changes in KEs (e.g., serum TH), and in turn how changes in TH concentrations may manifest in impacts on development and maturation, is incomplete. As quantitative data become available, additional modeling approaches to further characterize MIE-KE relationships in the thyroid AOP network could conceivably be implemented to predict thyroid-related adverse outcomes with associated uncertainty. Development of models that integrate HTS assay data for multiple MIEs will be an important step to enhance prioritization of chemicals for further screening and evaluation under the EDSP. For example, one of the challenges in evaluating endocrine responses to chemicals centers on understanding the time course of chemical effects. These quantitative challenges are not unique to the thyroid pathway and have been examined recently with chemicals that act on the ER and AR (Ankley and Villeneuve 2015). The absence of an apical outcome does not necessarily negate the predictive value of an upstream assay. Rather, it may be caused by testing for an endpoint too early in the time course or at a dose that was too low to elicit the adverse outcome. The converse can also be true whereby adverse outcomes are observed without observations of upstream KEs.

Conclusions

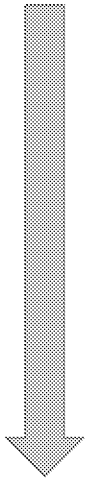
Several *in vitro* HTS assays are now available to help understand the potential for chemicals to interact with the thyroid axis pathway that will be helpful to for screening chemicals for thyroid activity. Because MIEs targeted by the existing and developing HTS methods were not included as part of the EDSP Tier 1 battery, relationships between the new HTS data being generated and existing animal-based data collected as part of the EDSP Tier 1 screening battery or from the published literature are discussed in this work. An AOP network describing the relationships between known thyroid-related AOPs and endpoints measured in EDSP Tier 1 assays is presented to demonstrate how new assay data and models may be used in prioritization and WoE applications.

Tables

Table 1. U.S. EPA Endocrine Disruptor Screening Program (EDSP) battery of 11 Tier 1 assays to screen chemicals for estrogen, androgen, and thyroid bioactivity, and Tier 2 assays for identifying adverse effects and dose-response relationships. Consistent with Adverse Outcome Pathways (AOPs), levels of increasing biological complexity from molecular interactions to individual and population level effects can be represented by the Tier 1 and 2 assays with the exception of the thyroid pathway, which lacks coverage of toxicity MOAs. E+ = estrogenic, E- = Anti-estrogenic, A+ = androgenic, A- = anti-androgenic, HPT axis = hypothalamic pituitary thyroid axis, EOGRT = extended one generation reproductive test, MEOGRT = medaka extended one generation reproductive test, LAGDA = larval amphibian growth and development assay, JQTT = Japanese quail toxicity test. Asterisk indicates EPA test guidelines harmonized with the OECD.



533 **Table 2.** Estrogen, androgen, and thyroid hormone lifecycles.



Lifecycle	Estrogen	Androgen	Thyroid Hormone
Biosynthesis	Steroidogenesis (e.g., P450scc, aromatase, 17β-HSD, 5α-reductase)		Iodine, NIS, TPO, IYD, pendrin, DUOX
Plasma Transport	SHBG, albumin		TTR, TBG, albumin
Homeostasis	GnRH, LHr, FSHr		TRHr, TSHr, Deiodinases, MCT8, MCT10, OATP1C1
Receptor	ER	AR	TR
Catabolism and Excretion	SULTs, UDPGTs, MRPs, p-glycoproteins (Mdr1)		

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535 17β-HSD = 17β-hydroxysteroid dehydrogenase; AR = androgen receptor; DUOX = Dual oxidase; ER =
536 Estrogen receptor; FSHr = Follicle stimulating hormone receptor; GnRH = gonadotropin releasing
537 hormone; IYD = Iodotyrosine deiodinase; LHr = Luteinizing hormone receptor; MCT = Monocarboxylase
538 transporter; NIS = Sodium-iodide symporter; OATP = Organic anion transporter polypeptide; P450 scc =
539 P450 enzymes, cholesterol side-chain cleavage; SHBG = Sex hormone binding globulin; SULTs =
540 sulfotransferase; TBG = Thyroid binding globulin; TTR = Transthyretin; TRHr = Thyrotropin releasing
541 hormone receptor; TPO = thyroperoxidase; TR = thyroid hormone receptor; UDPGT = Uridine
542 diphosphate glucuronosyltransferase.

Table 3. Identification of potential molecular targets of chemical-induced thyroid bioactivity. The toxicological relevance is characterized by current evidence that the molecular interaction regulates important biological processes and whether it has shown to be perturbed by environmental chemicals (Murk et al. 2013; OECD 2014).

MIE	Toxicological Relevance*	HTS Technology Status
TRH Receptor	Controls synthesis and release of TSH; TRH mutations lead to hypothyroidism. No reports of environmental chemical impacts although research is limited.	Existing
TSH Receptor	Controls thyrocyte functioning; TSH mutations can cause hypo- and hyper-thyroidism. No reports of chemical impacts although research is limited.	Existing
Sodium-Iodide Symporter (NIS)	Regulates iodide uptake in thyroid and is critical for TH synthesis. Inhibition of NIS-iodide transport impacts TH synthesis. Known target for environmental chemicals.	Existing
Pendrin	Gene mutations of pendrin can cause hearing loss, vestibular weakness, and sometimes goiter, but not necessarily hypothyroidism. No reports of environmental chemical impacts although research is limited.	R&D
Dual Oxidase (DUOX)	Generates peroxide necessary for TH synthesis; DUOX mutations result in organification defects and congenital hypothyroidism. No reports of environmental chemical impacts although research is limited.	R&D
Thyroperoxidase (TPO)	Only enzyme that catalyzes formation of THs. TPO is known target for xenobiotics, and there is a well-accepted AOP based on TPO inhibition as the MIE.	Existing
Serum Transport Proteins	TTR and TBG are major serum transporters of TH in vertebrates. TTR mutations alter TH kinetics and lower brain levels of T4. TTR and to a lesser extent TBG, can competitively bind chemicals.	Existing
Membrane Transporters	MCT8, MCT10, and OATP1C1 regulate cellular availability of TH. MCT8 mutations produce hypothyroidism and severe neurological impairments. Limited evidence suggests that some chemicals may impact their expression and functioning.	Promising
Iodothyronine Deiodinase (DIO)	Control TH homeostasis and the activation and inactivation of T4 in a tissue-specific and temporal manner. Chemicals can directly impact DIO functioning.	Promising
Iodotyrosine Deiodinase (IYD)	Scavenges iodide in the thyroid by catalyzing deiodination T1 and T2. Mutations in IYD result in hypothyroidism. Limited evidence that chemicals impair IYD; may be more important when dietary iodine is low or concomitant exposure to NIS inhibitors	R&D
Hepatic Nuclear Receptors (NRs)	Mediate Phase 1, 2, and 3 metabolism and disposition of endogenous and exogenous chemicals, including environmental chemicals, and contribute the TH homeostasis. Known molecular targets for a wide variety of environmental chemicals.	Existing
Sulfation and Glucuronidation	Sulfation and glucuronidation are important hepatic and nephric pathways that regulate TH catabolism. Known molecular targets for a wide variety of chemicals and are a well-accepted AOP for this based on this MIE.	Promising
Alanine Side-Chain	Alanine side-chains of T4 and T3 can be metabolized by oxidative decarboxylation or deamination; deamination produces thyroacetic acids and decarboxlation produces thyroanimines. Biological consequences of inhibiting are not well-described. No reports of environmental chemical impacts.	R&D
TH Receptor	T3-activated transcription factors of which there are two major subtypes TRα and TRβ that show tissue-specific and temporal functioning. Some chemicals bind to TRs. However, screens of large chemical libraries have revealed a low frequency hit rate, suggesting that binding may be restricted to a limited set of structures.	Existing
TH Transcription	Many TH signaling pathways are mediated by transcription of TR responsive genes and are critical to normal development and organ system functioning. Some chemicals may modify transcription by altering TR binding, co-factor recruitment, or TRE binding. Screens of large chemical libraries have revealed a low frequency hit rate, suggesting that perturbed transactivation may be restricted to a limited set of structures.	Existing

* Toxicological relevance is characterized by evidence that the MIE regulates biologically important processes that when disturbed can lead to adverse outcome(s), as well as any evidence that chemicals interact with the MIE.

Figures Legend

Figure 1. An Adverse Outcome Pathway (AOP) that is initiated with a molecular initiating event (MIE) and terminates in an adverse outcome (AO) that is linked by a series of intermediate key events. AOs at the organism level are used in human health risk assessment, and typically with plausible linkages (dotted line) at the population level for use in ecological risk assessment. Toxicity Pathways may be encompassed in an AOP and include MIEs and KEs that are plausibly linked to downstream apical responses.

Figure 2. Generalized overview of TH regulation and signaling in vertebrates with points along the central hypothalamic-pituitary-thyroid (HPT) and peripheral axes that have shown to be perturbed by environmental chemicals (red stars). TRH = thyrotropin releasing hormone; TSH = thyroid stimulating hormone; T4 =thyroxine; T3 = 3,3′,5-triiodothyronine; rT3 = 3,3′,5′-reverse T3; T2 = 3,3′-diiodothyronine; T1 = monoiodothyronine; UDPGT = uridine diphosphate glucuronosyl transferase; SULT = sulfotransferase; TH-G = glucuronidated thyroid hormone; TH-S = sulfated thyroid hormone; Mrp = multidrug resistance associated protein; Mdr1 = multidrug resistance protein 1 or P-glycoproteins; MCT = monocarboxylate transporter; OATP = organic anion transport polypeptide; TR = thyroid hormone receptor; RXR = retinoic x receptor.

Figure 3. Adverse Outcome Pathway (AOP) network for chemically induced thyroid bioactivity showing the integration of multiple individual AOPs under development and proposed. Biological linkages described may be informed by *in vitro*, *in vivo*, or computational data, and may be causal, inferential, or putative depending on the strength of the evidence. Boxes with red borders represent junctures and apical responses that are targeted by the EDSP Tier 1 screening battery, and showing that no MIEs are covered by the Tier 1 battery. Boxes with purple borders represent current MIEs with *in vitro* high-throughput screening (HTS) assays that have demonstrated reliability and are available for use in EDSP thyroid bioactivity screens; ToxCast assays are denoted with asterisks. Solid lines represent established linkages with quantitative or semi-quantitative data. Dotted lines represent plausible linkages with limited evidence. **HTS assays targeting D1 binding are further along in development than those targeting D2.

Figure 4. Generalized AOP network (putative) for the thyroid bioactivity pathway integrating *in vitro* high throughput screening (HTS) assays and *in vivo* EDSP Tier1/2 battery. The AOP network commences with any of several molecular initiating events (MIE) that can be predicted by emerging HTS assays with potential downstream linkages to intermediate key events (KEs) that culminate in adverse outcomes (AOs) identified *in vivo* EDSP testing. TRHr = thyrotropin releasing hormone receptor; TSH = thyroid stimulating hormone receptor; T4 =thyroxine; T3 = 3,3′,5-triiodothyronine; UDPGT = uridinediphosphate glucuronosyl transferase; SULT = sulfotransferase; NIS = sodium-iodide symporter; AMA = amphibian metamorphosis assay; EOGRT = extended one generation reproductive test; LAGDA = larval amphibian growth and development assay; JQTT = Japanese quail toxicity test.

585 # Figures

586 **Figure 1**

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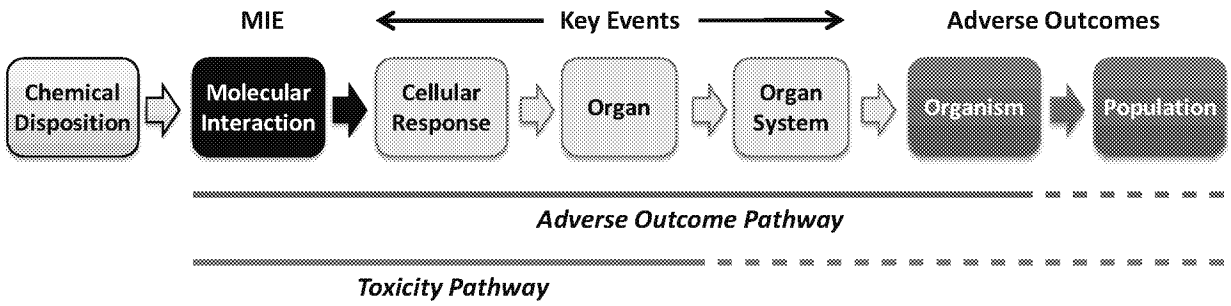

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589 **Figure 2**

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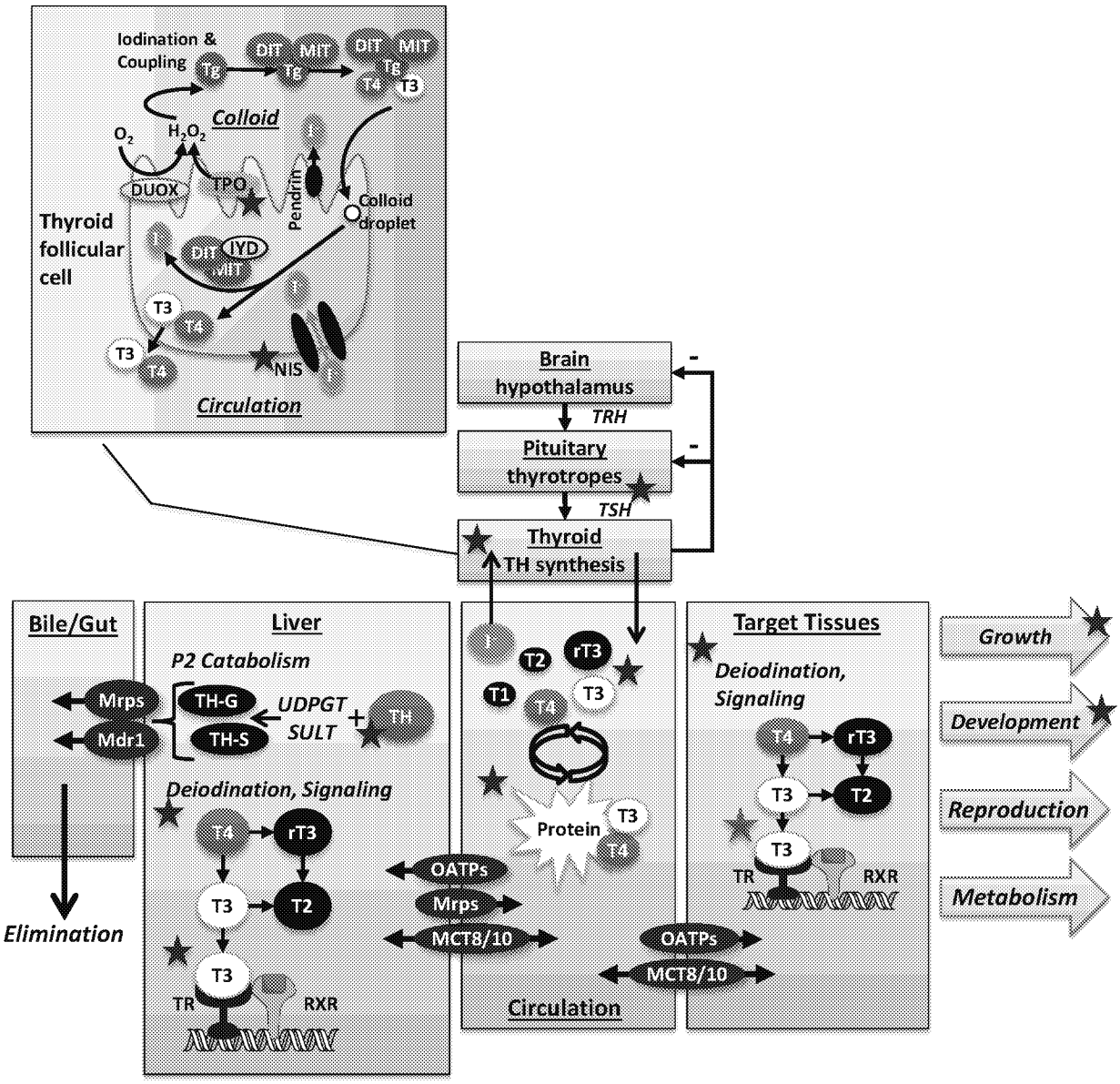

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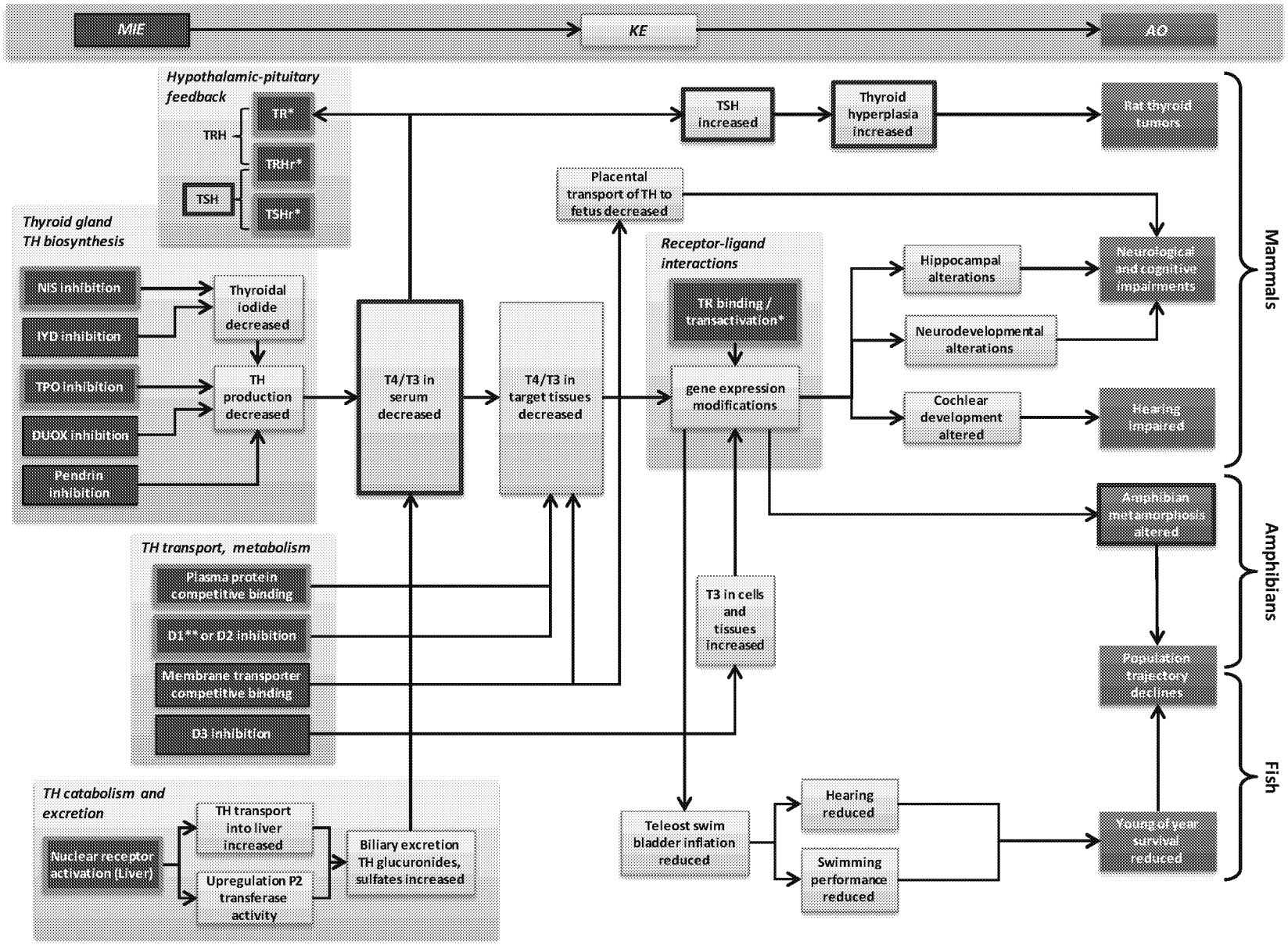
593 **Figure 3**



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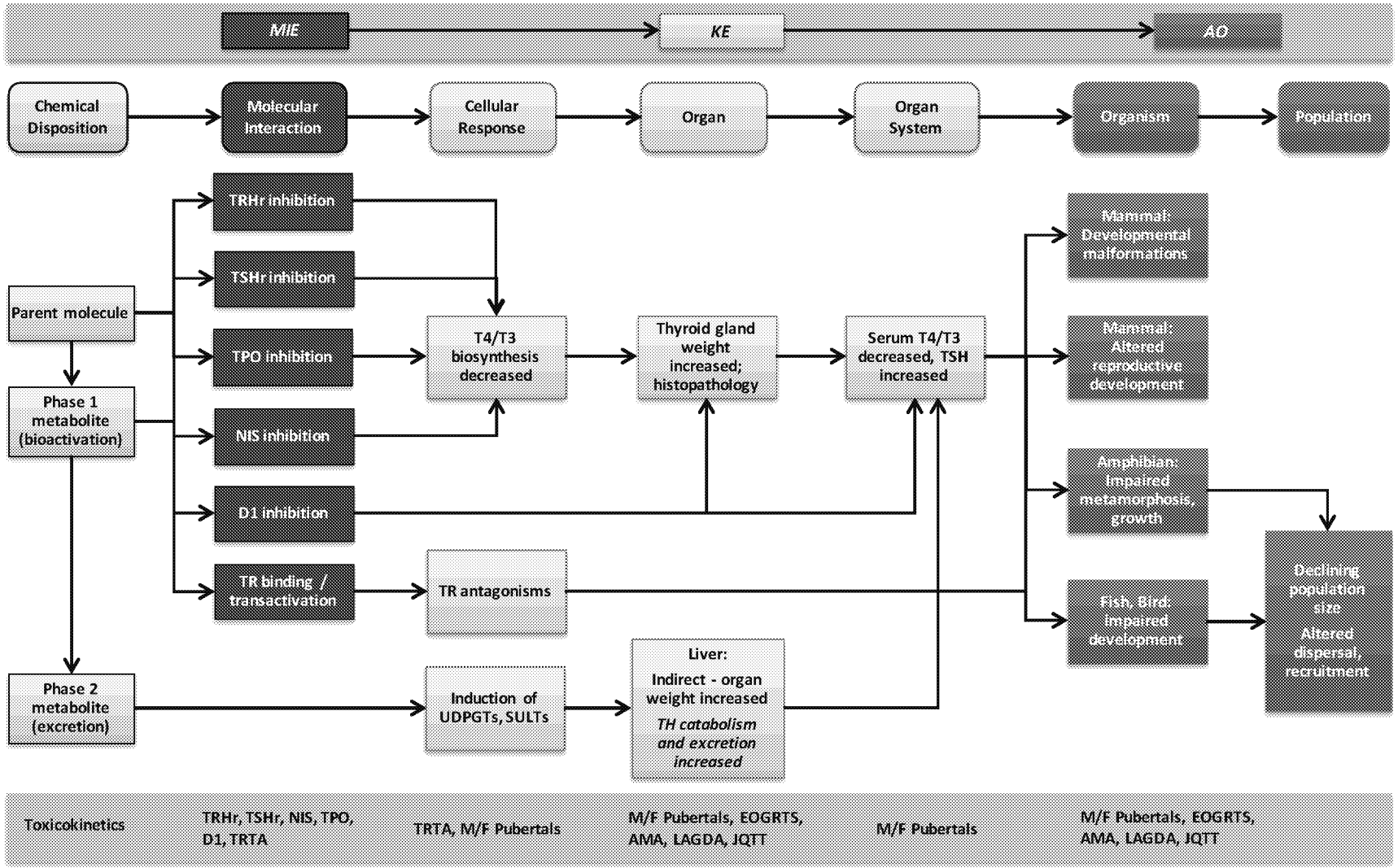
595 **Figure 4**



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